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The involvement of bacteria in the progression of Barrett's oesophagus to adenocarcinoma of the oesophagus

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Katie Blackett

2010

University of Dundee

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The involvement of bacteria in the progression of Barrett's oesophagus to adenocarcinoma of the oesophagus

By

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BSc (Hons.) (Microbiology, University of Dundee)

PhD Thesis
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Abbreviations

ADC	Adenocarcinoma
Bcl-2	B-cell lymphoma-2
BO	Barrett's oesophagus
bp	Base pair
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CHO	Chinese hamster ovary cell line
CLO	Columnar lined oesophagus
CLSM	Confocal laser scanning microscopy
CpG	Cytosine-phosphate-guanine dinucleotide
COX-2	Cyclooxygenase-2
dH_2O	Distilled water
DMEM	Dulbecco-modified eagle medium
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetra-acetic acid
EPS	Exopolysaccharide
FAME	Fatty acid methyl ester
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
GI	Gastrointestinal tract
GORD	Gastro-oesophageal reflux disease
HGD	High grade dysplasia
HH	Hiatal hernia
hTERT	human Telomerase Reverse Transcriptase

IM	Intestinal metaplasia
kDa	kilo Dalton
LGD	Low grade dysplasia
LOS	Lower oesophageal sphincter
LPS	Lipopolysaccaride
LB	Luria-Bertani
mA	Milliamp
MDM2	Murine double minute 2 (oncogene)
MOI	Multiplicity of infection
NK cell	Natural killer cell
NO	Nitric oxide
NQ01	NADP (H): quinone oxidoreductase 1
OGJ	Oesophagogastric junction
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGE2	Prostaglandin-E2
PI	Propidium iodide
PPI	Proton pump inhibitor
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
SOC/B	Super optimum culture/broth
STI	Soybean trypsin inhibitor
TLR	Toll-like Receptor
V	Volts

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Declaration

This thesis has been composed in its entirety by the candidate undersigned and has not been previously accepted for a higher degree. Unless otherwise acknowledged the work described here has been carried out by the candidate. All sources of information have been distinguished by references, all of which have been consulted by the candidate.

Signed,

Katie Blackett

Summary

Barrett's oesophagus (BO) arises from chronic gastro-oesophageal reflux disease (GORD). Patients have an increased risk of adenocarcinoma (ADC), which is the sixth most common cause of cancer mortality in the UK. All ADC develop from BO, and over the last twenty years there has been a marked increase in both conditions. The reasons for this are not known, however, as with some forms of gastric cancer, it is possible that there may be a bacterial aetiology. This study employed both culture-based and molecular techniques to characterise microbial communities colonising the distal oesophageal mucosae in individuals with GORD, BO and ADC, together with healthy controls. Furthermore, *in vitro* models were designed to create an oral microbiota, from which an oesophageal community could develop.

Microbial analysis identified a shift in oesophageal population composition with disease progression, with an incremental increase in total eubacterial scores related to the metaplasia-dysplasia sequence. Additionally, an increased proportion of Gram negative species and potentially pathogenic organisms, such as *Peptostreptococcus* were identified. *Campylobacter* spp. were isolated from 75%, 50% and 60% of GORD, BO and ADC patients, respectively, compared with 20% of controls. *Helicobacter pylori*, which has been proposed to be protective in oesophageal disease, was significantly reduced in disease, especially in ADC patients. *In vitro* models were successful, with a simple oral microbiota leading to the development of unique, varied oesophageal populations representative of those found *in vivo*. Additionally, after exposure of this community to bile acid, population dynamics were altered, with an increase in Gram negative species, associated with a rise in haemolytic and mucinolytic activities. Exposure of oesophageal cell lines to these

stressed biofilms resulted in increased cell death, and in some cases, amplified expression of p53 and COX-2.

In conclusion, this research proved an association between bacterial composition and oesophageal disease. With progression to adenocarcinoma, the community becomes increasingly diversified and Gram negative in character, and therefore, is proposed to be more pathogenic. Further research is required to investigate causal relationships, through which mechanisms for disease initiation and/or maintenance can be understood.

Chapter 1

Introduction

1.1 Introduction

Barrett's oesophagus (BO) is an acquired metaplastic change occurring after prolonged exposure of the lower oesophageal epithelium to refluxate due to gastro-oesophageal reflux disease (GORD).

In the United States, 20% of the population suffer from GORD, having the highest direct annual costs for healthcare compared to any other gastrointestinal disorder (Locke *et al.*, 1997; Sandler *et al*., 2002). Between 5 and 12% of patients undergoing endoscopy for GORD will be diagnosed with BO (Falk, 2002), and 5-10% of BO patients will go on to develop adenocarcinoma, at a risk of 0.5% per year (Blot *et al.*, 1991; Shaheen *et al.*, 2000; Solaymani-Dodaran *et al.*, 2003). Therefore, much of the research in this area has been concerned with factors that lead to GORD, and how the condition may be prevented.

The British Society of Gastroenterology has used a definition reviewed by a number of specialists in this area; an appropriate diagnosis views BO as “an oesophagus in which any portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium which is visible macroscopically” (Watson *et al.*, 2005).

In 2010, Cancer Research UK stated that cancer of the oesophagus was the ninth most prevalent cancer in the UK, a dramatic increase after formally being the twentieth. However, in men it is the sixth most prevalent cancer, with 5,000 new diagnoses per year (CRUK, 2010). In June 2006, the Scottish Intercollegiate Guidelines Network (SIGN, 2006) published new data showing that collectively, gastric and oesophageal cancers account for 6.5% of newly diagnosed cancer cases,

with particular problems in Scotland, and specifically, Tayside (SIGN, 2006). Of greater concern, 5% of cancer deaths are due to oesophageal cancer, being the sixth most common cause of cancer death in the UK (CRUK, 2010).

The majority of BO-associated adenocarcinomas are detected at an incurable, advanced stage of disease; therefore, the development of new treatments is urgently required. Studies show that patients in a proper surveillance regime have significantly increased survival rates upon detection, possibly due to their increased suitability for oesophageal resection (Fitzgerald *et al.*, 2001).

1.2 Barrett's oesophagus and cancer

1.2.1 Anatomy and physiology of the upper GI tract

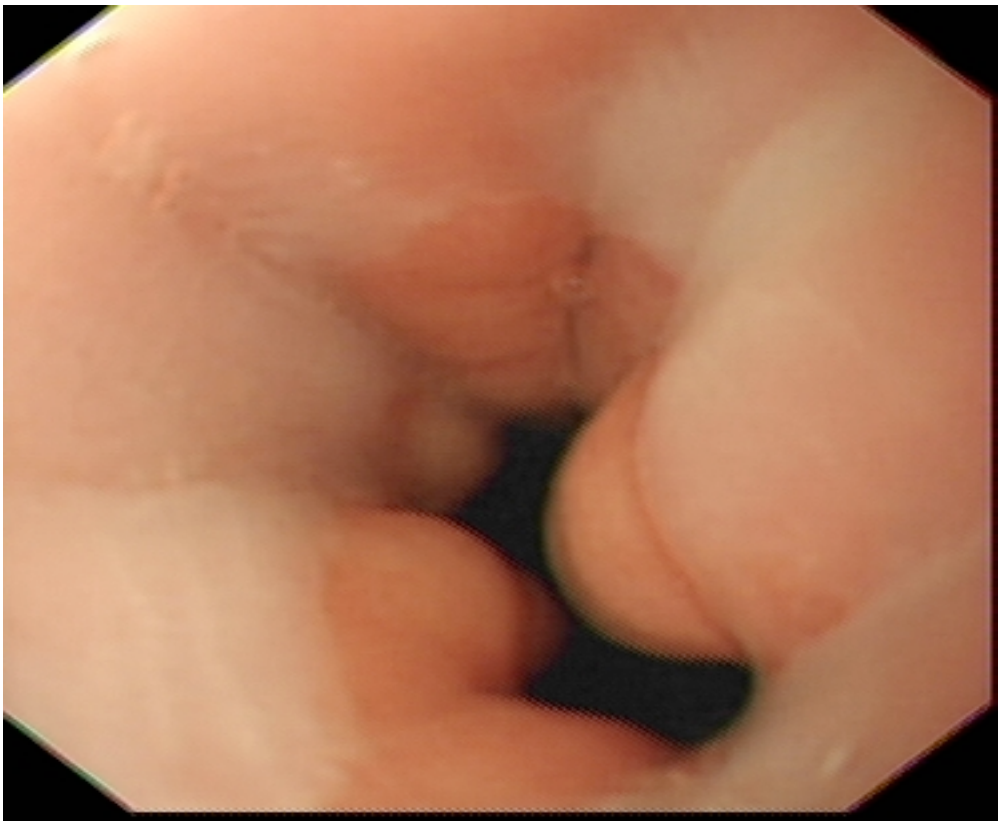


Fig. 1.1: *Endoscopy image of the oesophagus, showing the oesophagogastric junction (this study).*

The oesophagus is the canal leading from the mouth to the stomach (Fig. 1.1). This structure is on average 23 cm in length, and facilitates the passage of food and liquids into the upper gut. The oesophagus has three layers: muscular, cellular and mucosal. The muscles of the upper part of the tube are striated fibres, which allow for the voluntary action of swallowing, whereas lower oesophageal muscles are smooth and involuntary. Waves of contraction of these two muscle types cause peristaltic movement for the swallowing of food bolus. Small papillae coat the surface of the mucus layer, which covers a thick epithelium of stratified squamous cells (Fig. 1.2A). Glands are also present throughout the oesophagus in the submucosa. These produce mucus, providing lubrication for food bolus movement, and protection against abrasion. The oesophagogastric junction has an opening, or sphincter, which links the oesophagus to the stomach. The epithelial lining changes abruptly from a stratified squamous morphotype in the oesophagus to glandular tissue, as seen in Fig. 1.2B. The change in epithelial cell type occurs up to 2 cm back from the junction, and is called the squamo-columnar junction, or Z-line. The junction prevents reflux of acidic gastric contents back into the oesophagus due to pressure maintained between the stomach and oesophagus (Pettersson *et al.*, 1980).

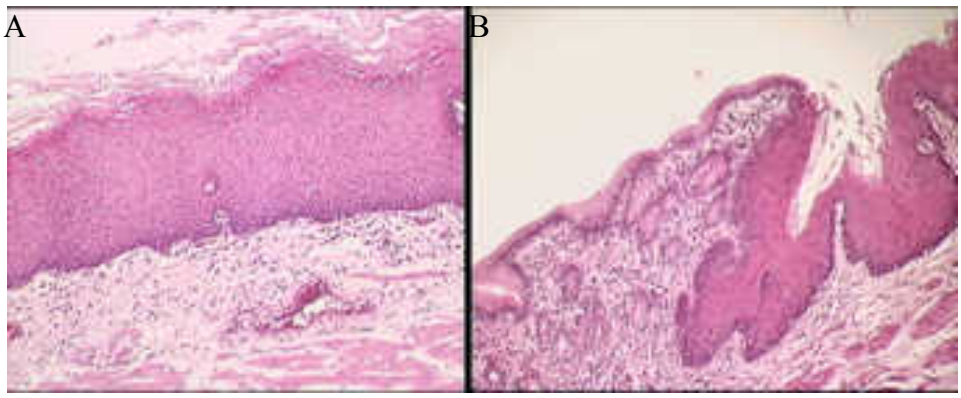


Fig. 1.2: *Histological images of the oesophagus. A: stratified squamous epithelium from the oesophagus, B: glandular epithelium of the oesophagogastric junction.*

1.2.2 Definition and history of BO

Barrett's oesophagus was first noted at the beginning of the last century (Tileston, 1906), where development of a columnar lined oesophagus was observed. Over the following 70 years, many papers were published describing the cellular morphology and hypothesising the causes of this condition. Dr Norman Barrett, a Consultant Surgeon at St. Thomas's Hospital in London initiated discussions, which would lead to the correct definition of this condition. He incorrectly described two separate conditions, which were later redefined by Allison and Johnstone (1953). Barrett had made two hypotheses, one describing the columnar lined oesophagus to be stomach lining drawn up into a congenitally short oesophagus, while the second proposed that the oesophageal lining had been abnormally altered by metaplasia (Barrett, 1950). Allison quickly published a paper positing his latter description to be correct (Allison, 1951). Since then a series of publications have refined this definition. Bosher's research described a patient with reflux oesophagitis whose mucosa contained goblet cells, more like those of the intestine, dismissing theories that this abnormal oesophageal lining could be from the stomach (Bosher and Taylor, 1951). Subsequently, Barrett published a small paper concurring with Allison, stating BO was not due to a congenitally short oesophagus (Barrett, 1957). A later study proposed the condition to be due to oesophagitis (Moersch *et al.*, 1959), which was verified in a study confirming the relationship between gastro-oesophageal reflux and metaplastic columnar lined oesophagus (CLO) (Hayward, 1961). Hayward also defined the oesophagogastric junction (OGJ) to be the end point of the oesophagus where it meets the stomach, noting columnar shaped cells to line 1-2 cm above and below this junction. Hayward also disproved Barrett's congenitally short oesophagus theory, stating that no babies had been found with this condition; therefore, it could

not be termed as congenital. However, confusion over this condition continued throughout the 1970s, until a large research study investigating biopsy tissue found three different types of Barrett's mucosa, mainly of intestinal origin (Paull *et al.*, 1976). Paull and co-workers recruited 11 patients, taking 112 biopsies from the oesophageal mucosa, and 39 from the gastric mucosa. Their research revealed varying clinical and histological features, including hiatal hernias, and three types of mucosa: functional, gastric and a specialised columnar similar to intestinal mucosa. This groups histological studies found no parietal cells in the CLO, but villiform folds and goblet cells representative of intestinal lining. The study affirmed the now accepted view of BO, or columnar lined oesophagus, being due to metasplasia after long-term reflux.

1.2.3 What is adenocarcinoma?

Cancer is the common term for a malignant neoplasm and accounts for 13% of all deaths, with 7.9 million deaths being due to this condition in 2007 (WHO, 2010). Carcinogens such as those in tobacco smoke, radiation, chemicals and infectious agents can initiate cancer. However, familial trends exist in many cancers, with genetic defects and mutations being inherited. A changed growth pattern may preside, resulting in overgrowth due to uncontrolled cell division. These tumour cells may invade adjacent cells and spread (metastasis).

Neoplasia, the abnormal proliferation of cells, facilitates subversion of the normal function of a cell, allowing clonal expansion to form a tumour. Cancer has a genetic basis, with functional errors relating to oncogenes (Huebner and Todaro, 1969) and tumour suppressor genes. Oncogenes destabilise controlled division, apoptotic

events and cell differentiation, conversely, tumour suppressor genes are anti-oncogenes; their related proteins are involved in cell cycle and apoptosis regulation. Epigenetics and genetics both play individual roles in cancer development; the latter includes changes in DNA sequence due to mutations and chromosomal rearrangements. Conversely, epigenetics involves DNA methylation and histone acetylation (Sharma *et al.*, 2010). Toxins or infectious agents generally cause epigenetic modifications, and it is thought they can be reversed after removal of these factors, and by treatment with enzyme inhibitors of methylation (Hussain and Harris, 2007). Of all epigenetic mechanisms, DNA methylation has been most extensively studied. This involves the addition of a methyl group to a cytosine ring of a CpG (cytosine-guanine with phosphodiester bond) dinucleotide. CpG dinucleotides are not evenly distributed along a genome, they are usually found gathered at the 5' end of a gene, and are termed "CpG islands" (Bird, 1986). These islands range in size from 400 to 4000 bp, and generally have a GC content of >55%, five-fold higher than usual bulk DNA (Takai and Jones, 2002). The University of California Santa Cruz Genome Bioinformatics Site has identified approximately 27,800 CpG islands in the human genome, with approximately 60% of genes containing these elements (Sato and Meltzer, 2006).

Epigenetic changes in gastric cancer

In gastric cancer, the frequency of aberrant CpG methylation is very high, with the number of methylated genes increasing significantly as disease progresses from gastritis to cancer along the metaplasia-dysplasia route (Kang *et al.*, 2003). *Helicobacter pylori* infection is the main aetiologic agent in gastric cancer, and its association with gastric epithelia results in changes in cytokine expression, and the

production of reactive oxygen species (ROS) and nitric oxide (NO) (Nardone and Compare, 2008). These factors are major causes of DNA methylation; therefore, it is believed that removal of *H. pylori* could reverse these epigenetic modifications.

The epithelial cell adhesin E-cadherin is a known factor in tumorigenesis, and in the invasiveness of cancer cells. A recent study by Chan and colleagues investigated the methylation status of E-cadherin in *H. pylori* positive and negative gastric cancer patients. Results showed that after eradication of this species, methylated E-cadherin was reduced, and the un-methylated form increased. Patients with persistent infection continued to have methylated protein, with two patients presenting with intestinal metaplasia. This research signifies the need for a fast response to this infection, because removal of these organisms could resolve epigenetic alterations and divert cancer progression (Chan *et al.*, 2006).

Table 1.1 identifies the main hypermethylated genes associated with gastric cancer, BO and adenocarcinoma of the oesophagus. Adapted from Sato and Meltzer, 2006.

Table 1.1: *Hypermethylated genes in gastric and oesophageal carcinogenesis* [a].

Gene	Full name/Funtion	GC	BO	ADC
APC	Adenomatous polyposis coli; Signal transduction	78%	80%	70%
CDH1	Calcium dependant adhesion molecule; Cell cycle regulation/maintains intercellular junctions in normal epithelia	61%; Metastasis and prognosis, usually diffuse type rather than IM	10%	70%; Lymph node metastasis
MGMT	O^6 methylguanine-DNA methyltransferase; DNA repair/ prevents G:A mutations and DNA strand breaks	23%	43%; HM frequency	59%; associated with poor prognosis
HPP1	Hyperplastic polyposis 1; Growth factor	50%	44%; malignancy potential for ADC	68%
TIMP3	Tissue inhibitor of metalloproteinase 3; Antiangiogenic factor	43%	60%; malignancy potential for ADC	56%
RUNX3	Transcription factor; TGFβ signalling pathway	65%; Cell proliferation; increases sequentially	25%	48%
p16/INK4A	Cell cycle regulation	38%; Intestinal type	20%	45%
p15/INK4B	Cell cycle regulation	58%	12%	4.5%
p14/ARF	Cell cycle regulation; inhibits p53 degradation	33%	4%	0%

[a] *Constructed from Sato and Meltzer (2006), review of DNA hypermethylation in gastric carcinoma (GC), Barrett's oesophagus (BO) and oesophageal adenocarcinoma (ADC).*

Lessons learned for oesophageal adenocarcinoma

Two types of oesophageal cancer exist, adenocarcinoma and squamous cell carcinoma. The incidence of adenocarcinoma has been increasing rapidly over the last three decades, especially in the Western world. In the UK, the prevalence of adenocarcinoma is now almost 50% of all oesophageal cancers (Watson, 2000). Squamous cell carcinomas are found in the upper third and middle oesophagus, originating in the epithelial lining, and are associated with alcohol intake and smoking damage (Ribeiro *et al.*, 2005). Adenocarcinoma is prevalent in the lower third of the oesophagus, originating in gland cells of the mucous lining, developing from Barrett's oesophagus as the result of acid reflux.

Gastric cancer is a good model on which to base our current perception of oesophageal adenocarcinoma. It is a cancer of the upper GI tract, with a progressive lineage from inflammation (gastritis, GORD), to intestinal metaplasia, leading to neoplasia and adenocarcinoma. As discussed in the previous section, epigenetic modifications of the genome are involved in gastric cancer, with similar findings in oesophageal ADC. The presentation of acidic stomach contents to the oesophageal mucosa results in oxidative stress, inducing the development of metaplasia (BO) and dysplasia. The protective enzymes peroxidase and glutathione S-transferases are mechanisms of defence against oxidative stress from ROS and NO, however, a study of 159 patient samples (normal, BO and ADC) found that these enzymes genes were silenced due to hypermethylation at their transcriptional start sites in diseased patients (Peng *et al.*, 2009). Figure 1.3 shows the changes involved in adenocarcinoma development.

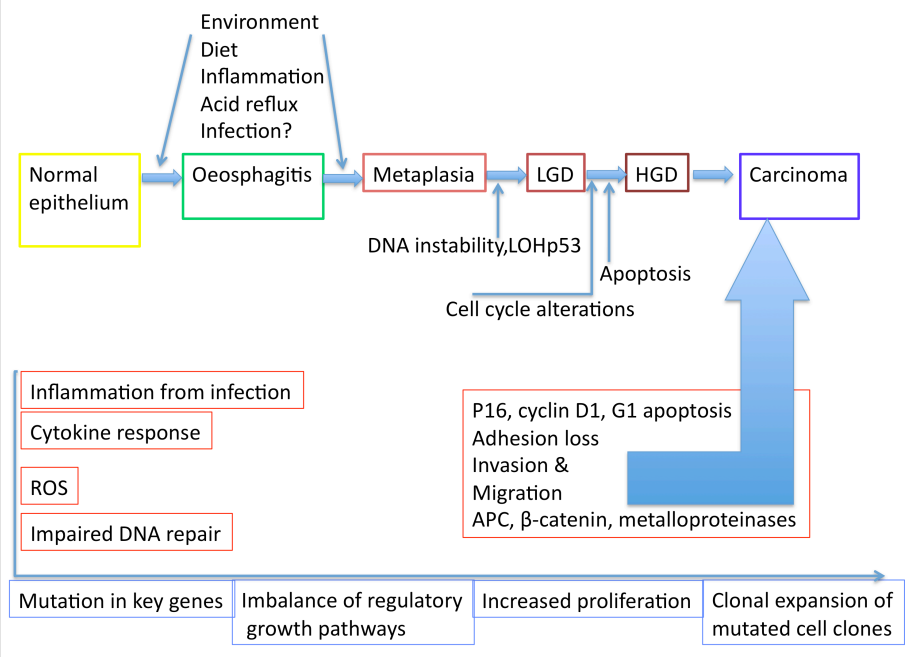


Fig. 1.3: *Progression of events resulting in oesophageal adenocarcinoma. Adapted from Manjunath and Jankowski (2000).*

1.2.4 Aetiology of GORD and BO

In comparison to the healthy oesophagus, the disease state associated with BO results from changes in the normal tissue function, and BO is a complication of gastro-oesophageal reflux. Prolonged reflux disease results in oesophagitis, where the oesophageal lining becomes inflamed, with peptic strictures. The prime suspects in oesophagitis development are the contents of refluxate washed up from the stomach, particularly gastric acid and bile salts. A defective lower oesophageal sphincter allows entry of gastric juices into the oesophagus. This can occur due to hiatal hernias, increased abdominal pressure, or as the result of obesity.

The lower oesophageal sphincter (LOS)

When LOS resistance is lost due to gastric distension, either permanently or transiently, reflux can occur. This usually occurs after a meal, and can happen in normal patients without GORD. However, if reflux persists, the mucosa becomes

inflamed resulting in peptic strictures, being described as reflux oesophagitis. The first publication detailing the process of GORD used newly developed X-ray equipment to study regurgitation in a cat (Cannon, 1911). In 1956, Fyke's group used oesophageal manometry to measure muscle pressure in the healthy oesophagus, demonstrating an intra-luminal high-pressure zone interposed between the oesophagus and stomach in humans, which is upheld by muscles of the distal oesophagus (Code *et al.*, 1956). In a healthy oesophagus, the OGJ acts as a barrier between the acidic stomach contents and the oesophagus. The LOS was also studied with standard manometry, with data from normal subjects being compared to those of 272 GORD patients (Zaninotto *et al.*, 1988). Results indicated that if the gastro-oesophageal sphincter relaxes and opens inappropriately it allows the backflow of stomach contents into the oesophagus, resulting in mucosal damage. Another patient-based study examined 322 patients with 24 hour oesophageal pH monitoring for exposure to gastric juices, and verified a defective LOS as a factor in the occurrence of GORD (Stein *et al.*, 1992). This group described a defective LOS with particular characteristics: less than 2 cm average length, and an average resting pressure of less than 6 mm Hg. The work showed two specific correlations: both defective LOS, and percentage time at pH less than 4, increased proportionally with mucosal injury.

Obesity and increased LOS pressure

As discussed previously, the incidence of adenocarcinoma has increased greatly in the last two decades, together with GORD and BO, which parallels the severe increase seen in obesity in western populations (Devesa *et al.*, 1998). A National Health and Nutrition Examination Survey (NHANES) in the US, comprising adults

aged 20-74 years, showed an increased prevalence of obesity (BMI >30) from 15% - 32.9% from 1976-2004 (Flegal *et al.*, 1998, 2002; Ogden *et al.*, 2006).

Obesity increases abdominal pressure (Barak *et al.*, 2002) along with other physiological changes which would cause reflux, such as reduced LOS pressure, increased frequency of sphincter relaxation and impaired gastric emptying (Hampel *et al.*, 2005). The relationship between GORD and obesity appears to involve an increased gastro-oesophageal sphincter pressure gradient between this structure and the stomach (Mercer *et al.*, 1987), intra-abdominal pressure (El-Serag *et al.*, 2006) and hiatal hernia (Pandolfino *et al.*, 2006). A study of 1224 patients undergoing an upper endoscopy, showed these obese patients were more likely to have a hiatal hernia and oesophagitis compared with lean individuals (Berstad, 1988). Additionally, impaired gall bladder and pancreatic function is more common in obese patients, resulting in an altered enzymatic composition. Wisen *et al.* (1988) found that output levels of pancreatic enzymes and bile were higher in the obese. If obesity is a risk factor for developing GORD, then diet must also be implicated. Many studies have shown that a decrease in fruit, vegetables, vitamins and fibre intake, accompanied by an increase in fat, will substantially increase the possibility of developing ADC due to GORD (Veuglers *et al.*, 2006).

Hiatal hernia

Proposals for the involvement of hiatal hernias (Fig. 1.4) in GORD have also been explored (Allison, 1951) separate to obesity. There involvement is due to a distortion in oesophageal anatomy, which impairs normal peristalsis.

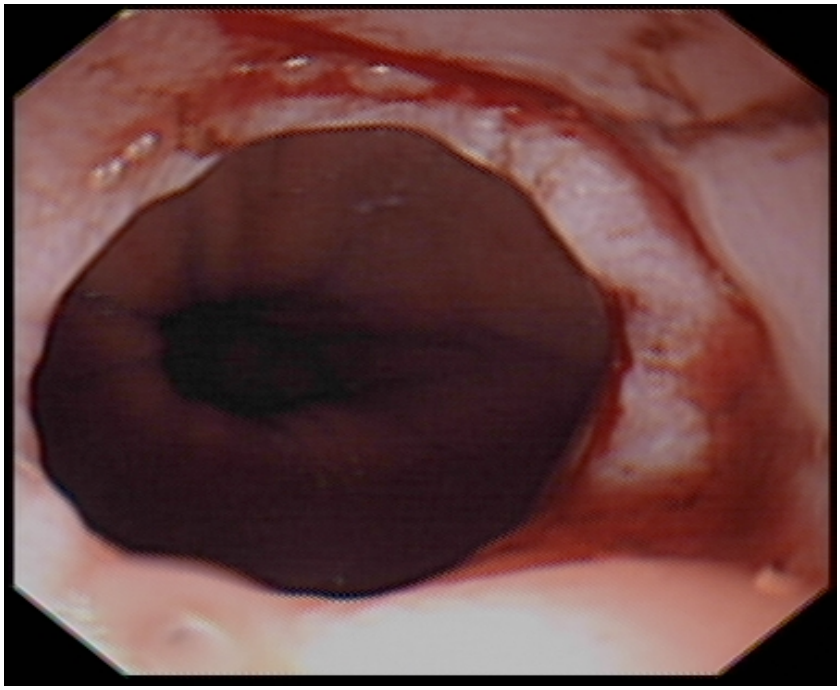


Fig. 1.4: *Endoscopic image of a hiatal hernia (this study).*

A patient study showed those with large hiatal hernia (HH) to have weaker and shorter LOS, compared to those with a small or no hernia (Patti *et al.*, 1996). A subsequent investigation scrutinised 375 patients with pH monitoring (Fein *et al.*, 1999). These investigators recruited patients with hiatal hernias, a defective LOS or both, into the study. Results showed that each factor seemed to be associated with GORD and its progression, and that collectively, the effects were intensified. Two recent patient studies reported that 63% of those with GORD-related BO had hiatal hernias (Westhoff *et al.*, 2005), while those with longer segments of BO had a larger HH (Dickman *et al.*, 2005).

Stomach acids and bile salts

In 1884, Reichman lowered a sponge into the oesophagus of a patient with heartburn and detected acid on its retrieval, postulating that gastric acid was involved in oesophagitis (Bennet, 1987). Most studies have emphasised that acid from the

stomach is mainly responsible for inflammation, and although bile is also implicated, these investigations were limited due to the lack of suitable experimental methodologies (Nehra *et al.*, 1999). However, a new monitoring system for bilirubin was developed in the late 1990s, and Oh *et al*. (2006) employed this method to evaluate gastric acid and bilirubin exposure to the oesophagus over a 24 hour period. Their study showed that the combination of gastric acid and bile produced more severe mucosal injury than acid alone.

Recurrent or continuous reflux of acidic gastric contents results in chronic mucosal damage of the lower oesophagus, which leads to a protective metaplastic alteration to the epithelial lining. These columnar cells are more resistant to acidic conditions, and are representative of BO.

1.2.5 Pathophysiology of oesophageal disease

Reflux disease: NERD and GORD

A common misconception is that reflux (heartburn) and GORD are the same, however, they are two separate conditions. Reflux can nevertheless result in GORD, where erosion of the mucosa causes lesions in the oesophageal lining, while in some cases there is no obvious erosion, as in non-erosive reflux disease (NERD). GORD is considered a predisposing condition for ADC, due to its induction of BO, and is one of the most common conditions in the USA, leading to symptoms in 40% of the population every month (7% per week) (Gallup Organisation 1988; Moayyedi and Axon, 2005).

Regurgitation of gastric contents may also manifest as pulmonary disease (Peters *et al.*, 1998; Jailwala and Shaker, 2000; D'Urzo and Jugovic, 2002), dental erosions (Jailwala and Shaker, 2000), reflux laryngitis (Catalano *et al.*, 2004), or non-cardiac chest pain (Davies *et al.*, 1985; Richter *et al.*, 1989; Eslick *et al.*, 2003). The latter is generally known as heartburn; however, its medical name is pyrosis, which is the most common symptom of reflux disease. Of patients presenting with reflux disease, it is approximated that 70% have NERD or endoscopically negative reflux disease (Martinez *et al.*, 2003). These patients are less likely to respond to standard anti-reflux therapy, possibly because the majority have normal ambulatory 24 hour pH tests. A study by Shi *et al.* (1995) reported on 771 patients who underwent 24 hour pH monitoring due to GORD symptoms, non-cardiac chest pain or both. Endoscopy allowed histological analysis of the oesophagus, and BO patients were excluded. This study showed that patients with a normal oesophagus and normal acid exposure could still have simultaneous reflux symptoms, although these were frequently related to a longer exposure with pH <4. These patients may have an 'acid-hypersensitive oesophagus', since many patients with GORD and oesophagitis had symptom-free episodes. Therefore, NERD patients could benefit from treatments that reduce oesophageal sensitivity (Marrero *et al.*, 1994), rather than reducing acid secretion.

Barrett's oesophagus and adenocarcinoma

Prolonged exposure of normal oesophageal squamous cells above the OGJ to gastro-duodenal refluxate can result in inflammation of the mucosa. These injured mucosal squamous cells undergo repair, and in a number of cases, are replaced by columnar epithelium, which are better able to withstand the low pH environment (Spechler,

2002). Biopsies taken from BO mucosae (Fig. 1.5) in the past were confused with gastric cells due to their columnar appearance. However, histologists can now distinguish tissue of oesophageal origin, due to the presence of glands and crypts situated in the mucosa representative of intestinal mucosa.

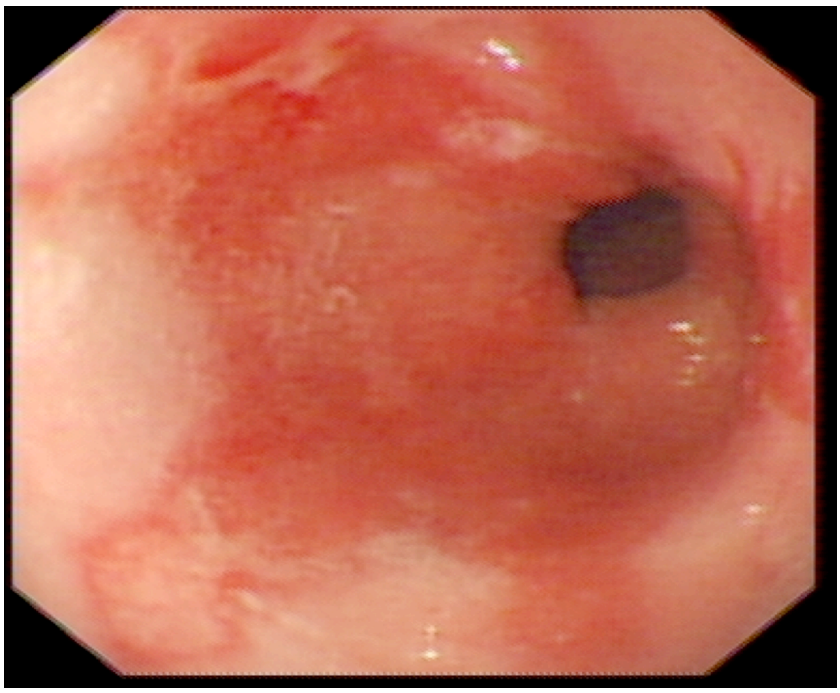


Fig. 1.5: *Endoscopic image of intestinal metaplasia from a patient with BO (this study).*

It is logical to conclude that as GORD symptoms and duration increase, Barrett's severity would also increase proportionately. However, this does not seem to be the case, with the length of BO segment being typically established in only a few months (Cameron and Lomboy, 1992).

If oesophageal cells continue to differentiate and proliferate, becoming more irregular and disorganised (dysplasia), tumours may develop resulting in adenocarcinoma of the oesophagus (Fig. 1.6) (Nandurkar and Talley, 2000) and ultimately death. This connection was first postulated in the early 1950s (Carrie,

1950; Bosher and Taylor, 1951; Morson and Belcher, 1952), due to the strong interest in BO and its connections to reflux. Morson and Belcher (1952) prepared a case report on a single patient with dysphagia (difficulty swallowing), taking biopsies from an ulcerated lesion found during gastro-oesophageal endoscopy. There were no other abnormalities in the lower oesophagus or gastric tissue, however, histology showed the lesion to be of intestinal origin, with many goblet cells, chronic inflammation and atrophy, that is, Barrett's oesophagus.

As with BO, many publications then appeared with studies looking at biopsies associating BO to adenocarcinoma (Adler, 1963; Hawe *et al.*, 1973; Haggitt *et al.*, 1978). In 1987, Reid posited intestinal metaplasia to predispose for tumour development (Reid and Weinstein, 1987), and it was subsequently confirmed that this intestinal metaplasia was the most common and clinically important with respect to BO leading to ADC (Spechler, 1994).

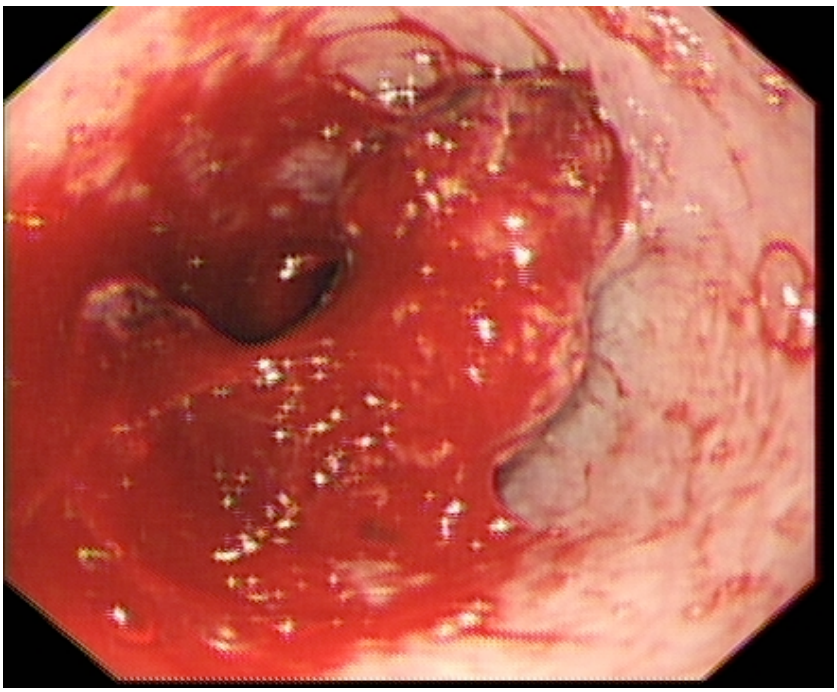


Fig. 1.6: *Endoscopic image of a cancerous lesion from a patient with oesophageal adenocarcinoma (this study).*

1.2.6 Clinical investigations

Upper gastrointestinal endoscopies allow visualisation of the oesophagus with pinch samples (biopsies) taken for histology and research purposes. The documented increase in BO and ADC prevalence is alarming, however, whether this is a true increase, or is due to increased endoscopy investigations is unclear. In Tayside, where the incidence of GORD and BO is the highest in Scotland, upper GI endoscopies increased by 168% between 1980 and 1995, with newly diagnosed oesophagitis rising by 117% (a decrease of 18% per 1000 procedures), and Barrett's by 4533% (Todd *et al.*, 2002). Studies by Conio *et al.* (2001) and Irani *et al.* (2005) showed a correlation between increased incidence and endoscopy use, whereas conflicting data from Prach *et al.* (1997) and van Soest *et al.* (2005) illustrate an independent increase in incidence. A 1996-2002 study by Musana *et al.* (2008) considered only histologically confirmed BO, and found a prevalence of 0.26% in a rural, non-referral population from Wisconsin. They also found that the prevalence rate increased with age, with a higher incidence in men. Over this seven-year period, incidence rates for initial diagnosis remained constant, despite the increase in endoscopy procedures. With a definite increase in incidence of adenocarcinoma of the oesophagus, it seems likely that GORD and BO incidence is also rising, irrespective of improved screening, treatments and awareness.

Surveillance systems for identifying BO patients progressing to dysplasia are time consuming and expensive with a low yield, because neoplastic progression occurs as little as once per 200 patient years (Shaheen *et al.*, 2000). Therefore, an improved method for detecting "at risk" patients would be extremely beneficial, and much research into molecular biomarkers is currently underway. As discussed previously,

DNA methylation occurs early in the progression of BO to ADC, and therefore biomarkers for hypermethylation of specific genes could be effective in predicting neoplastic risk. Collaborative work by the Meltzer group at John Hopkins University has previously identified *p16*, *RUNX3* and *HPP1* hypermethylation in BO-associated ADC (Schulmann *et al.*, 2005). Further studies by this group for specific gene methylation in 259 patients identified hypermethylation in five other genes, which led to a double-blinded validation study to measure the accuracy of these biomarkers in predicting neoplastic progression (Jin *et al.*, 2009). Results showed that methylation values of *HPP1*, *p16* and *RUNX3* were higher in patients progressing to neoplasia compared with non-progressors, 50% of which would not have been diagnosed early without these biomarkers. This study model has the potential to reduce the number of endoscopic procedures needed, and increase the identification of "at risk" patients.

1.2.7 Treatments

Patients presenting with reflux symptoms are generally prescribed proton pump inhibitors (PPI), which reduce gastric acid secretion, thereby increasing pH. Studies have shown that these drugs reduce pain and can heal areas of oesophagitis (Katz and Zavala, 2010). No side effects have been confirmed to be directly associated with these drugs to date. However, bacterial overgrowth in the stomach due to increased pH may have delayed effects in diseases of the gastrointestinal tract and this needs to be further studied.

Post-diagnosis of BO, treatments are directed at preventing progression of disease to ADC, and the neutralisation of GORD symptoms. Drastic measures such as

fundoplicative surgery to reduce reflux have been compared with medicative treatments, showing no reduction in rates of adenocarcinoma progression (Corey *et al.*, 2003). PPI to prevent reflux do not shorten the length of existent BO (Sharma *et al*., 1997), although if used before BO development, can reduce reflux symptoms giving an increased prevalence of short segment BO (3.2 cm *vs.* 4.8 cm without PPI therapy) (El-Serag *et al.*, 2004). The NSAID, rofecoxib, was administered to 12 patients with BO and biopsies were taken before and after 10 days of therapy. After 10 days, COX-2 expression, prostaglandin-E2 (PGE2) content and proliferating cell nuclear antigen (PCNA, a marker of cell proliferation) expression were reduced in BO tissue, indicating this drug could be used as therapy for ADC prevention along with acid-suppression (Kaur *et al.*, 2002).

In an animal study by Oyama *et al.* (2005), 120 rats underwent a surgical duodenoesophageal reflux procedure; half were given standard chow, while the others were given celecoxib (NSAID). The animals were sacrificied at 10, 20, 30 and 40 weeks for real-time PCR analysis and immunohistochemistry of COX-2 expression, and protein levels, respectively. In the control group, GORD, BO and ADC developed over time, compared with the treatment group, where incidence of oesophagitis was reduced or mild with no ADC found. No significant differences were observed for COX-2 expression between groups however, PGE2 and Ki-67 (cell proliferation) were markedly reduced in the treatment group ($P<0.05$). These studies indicate a preferred role for NSAIDs/COX-2 inhibitors rather than PPI in preventing progression to BO and ADC if detected early, or used in conjunction, to reduce effects of reflux and minimize inflammation.

If disease is not caught early, ablation, argon plasma coagulation and photodynamic therapy are possibly the best treatments for high grade dysplasia, as these may be better tolerated than surgery and offer the chance of complete removal of dysplastic epithelia (Johnston, 2005). Although oesophagectomy offers the best possible success rates for obliteration of disease, the risks involved can be anywhere between 3% and 15% dependent on care facilities and volume of oesophagectomies performed (Swisher *et al.*, 2000). Additionally, ablation carries a risk of only removing surface epithelia while leaving metaplastic islands to develop below the healthy appearance of the mucosal cells, which can develop to ADC (Barham *et al.*, 1997). However, in patients with early stage cancer who are not good candidates for surgery, endoscopic resection, laser therapy and photodynamic therapy offer remission rates of 45-75% (Overholt *et al.*, 1999; Ell *et al.*, 2000; Van Laethem *et al.*, 2001; Wolfsen *et al.*, 2002).

1.2.8 The immune response

The intestinal mucosa and epithelium form a barrier to unwanted molecules and microorganisms, which is regulated by the innate and acquired immune system, and physical and chemical defences. Immune cells have an elaborately developed network of lymphoid tissue, with lymphocytes free in the mucosa and lymph nodes, and aggregated in follicles such as Peyer's patches. Mucosal antibodies (secretory IgA) and T cells play a highly significant role in these mucosal defence mechanisms. Innate responses do not require a previous exposure to infection, involving the activation of toll-like receptors (TLR), a type of pathogen-associated molecular pattern (PAMP) (Akira *et al.*, 2001). This initial line of defence results in induction of inflammatory mediators (cytokines, reactive oxygen species and NO). These have

significant anti-microbial effects, however, they must be tightly controlled to avoid development of chronic inflammation and damage. Stimulation of TLR can also lead to further recruitment of macrophages, dendritic cells, natural killer cells (NK cells) and the resultant development of an adaptive immunity to the antigen (Akira and Takeda, 2004).

In the case of *H. pylori* and gastric cancer, this bacterium and its LPS activate TLR, which induce cytokine production as part of the innate response. TLR upregulate genes for various cytokines such as IL-2, IL-3 and IL-12. Figure 1.7 represents the full immune response to *H. pylori* (Wilson and Crabtree, 2007). *Helicobacter pylori* also activates the adaptive immune response through macrophages and dendritic cell recruitment, which produce IL-12 and stimulate Th1 cells. Macrophages are involved in the production of IL-1, IL-6 and TNF-α, possibly resulting in further NF-kappa B pathway signalling (Pathak *et al.*, 2006).

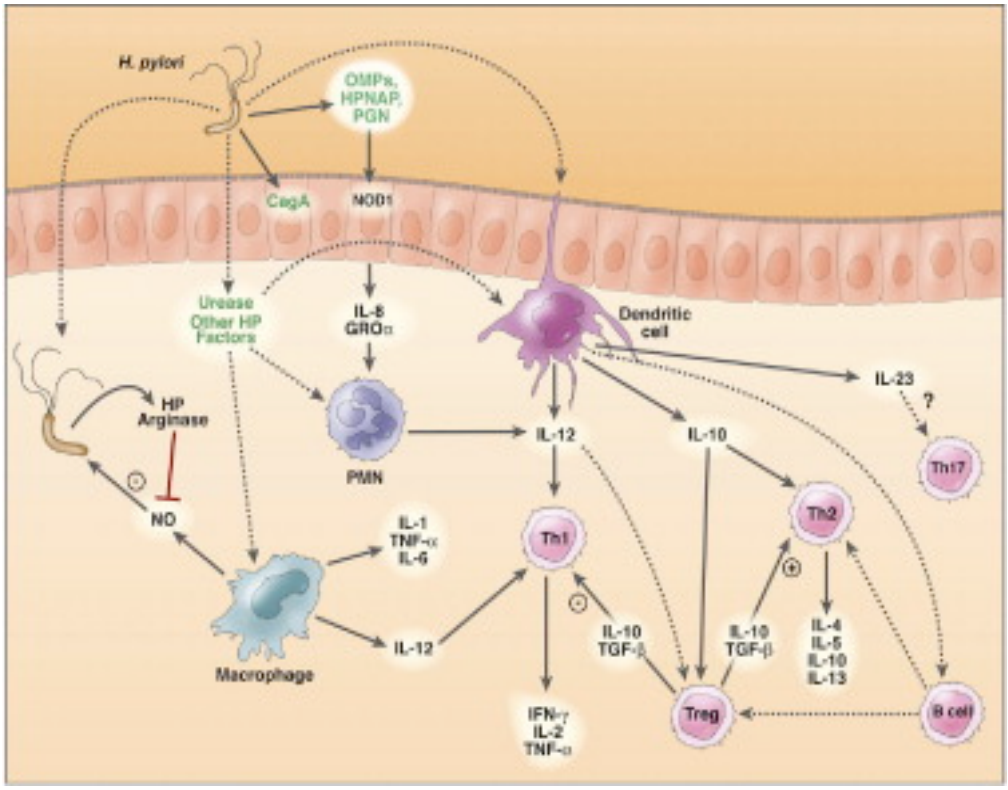


Fig. 1.7: *The immune response to H. pylori infection. Dotted lines are speculative components, items in green are of bacterial origin and black are host. (Wilson and Crabtree, 2007).*

Macrophages can also act as effector cells upon infection with this bacterium, producing NO via the enzymes inducible nitric oxide synthase (iNOS and iNOS2). However, *H. pylori* has adapted a number of evasive mechanisms. The organisms can release arginase, an enzyme that produces urea from host L-arginine, leading to ammonia production by urease. This neutralises HCl in the gastric lumen. However, arginase can also compete with macrophages for iNOS substrate (L-Arg), reducing production of NO and protecting the bacterium (Gobert *et al.*, 2001). Urease and outer membrane proteins also have the capacity to cause apoptosis of immune and epithelial cells, leading to reduction and dysregulation of the immune response. Therefore, *H. pylori* infection is multi-factorial, with inflammatory responses leading to disruption in pH homeostasis, which then allows continued colonisation by the organism and further immune cell invasion. *Helicobacter pylori* strains with the *cag*

pathogenicity island (PAI) further promote cytokine and chemokine responses, resulting in peptic ulcer development though a prolonged Th1 response (Hida *et al.*, 1999).

In the case of GORD and BO mucosal damage via bile and acid reflux recruits inflammatory cells such as TNF-α and IL-1β. Studies with TNF-α knockout mice reveal their resistance to skin carcinogenesis, implicating this cytokines involvement in tumourigenesis, possibly through direct mechanisms or the further recruitment of other cytokines and metalloproteinases (Tselpsis *et al.*, 2002). These workers investigated TNF-α expression in 40 BO patients, compared with 20 normal, 20 GORD, 20 dysplasia and 85 ADC biopsies. Results showed that TNF-α expression was increased throughout disease, with large areas of expression around glands in dysplastic areas of tissue. Moons *et al.* (2005) recruited 40 GORD and BO patients, revealing an increased Th2 response in BO patients compared with GORD. BO patients had higher levels of mast cells and IgG presenting plasma cells, confirming the progression to a humoral-based response as BO and ADC develop. This could be due to increased expression of COX-2, which inhibits the Th1 response and upregulates Th2 cytokines such as IL-4 and IL-10 (Fitzgerald *et al.*, 2002a). Two studies by Dvorakova and co-workers have investigated the role of IL-6 and its related pathways in BO. Initially, biopsies were taken from 15 patients with intestinal metaplasia, revealing increased production of IL-6 in IM tissue compared with duodenum or adjacent squamous epithelia (Dvorakova *et al.*, 2004). Later, the same group exposed the Seg-1 adenocarcinoma cell line to a bile acid cocktail at pH 4, resulting in increased STAT-3 activation and expression of IL-6 (Dvorak *et al.*, 2007).

A study by O'Riordan investigated the inflammatory response in GORD, BO and ADC patient biopsies using ELISA techniques (O'Riordan *et al.*, 2005). A correlation between disease progression and increased expression of NF-Kappa B, IL-1β and IL-8 was noted, however, NF-kappa B negative ADC patients had reduced levels of these two cytokines. The Fitzgerald laboratory in Cambridge recruited 50 patients with BO to investigate cytokine expression relating to the inflammatory gradient using ELISA. Biopsies were taken from IM segments and squamous epithelia in these patients. Proximal BO biopsies had increased expression of IL-8 and IL-1β compared with the distal BO segment, conversely, IL-10 expression was higher in distal tissues. Expression of IL-4 protein was increased in IM biopsies, compared with normal squamous mucosa (Fitzgerald *et al.*, 2002a).

The main pro-inflammatory cytokines implicated in cancer development are IL-1, IL-6, TNF-α, IL-15 and MIF (macrophage migration inhibitory factor). IL-6 has been shown to enhance and maintain hypermethylation of the p53 gene and decrease promoter methylation of the epidermal growth factor receptor (Hussain and Harris, 2007). COX-2 is also involved in infection-induced inflammatory responses, and prostaglandin production. Its over abundance or degradation can result in synthesis of mutagenic compounds (Hussain and Harris, 2007). The pro-inflammatory cytokine profile of BO and ADC is evidenced by these studies, with TNF-α, IL-1β, IL-4, IL-6 and IL-8 representing the major players. This mirrors the pattern seen in gastric cancer initiated by *H. pylori*, with the pro-cancer immune cells TNF-α and IL-6. In gastric cancer, the immune response to infection also depends on the hosts' genetics, and this may also be a factor in oesophageal adenocarcinoma, where mucosal biofilms may be involved in the inflammatory process.

1.3 Microbial colonisation of the healthy upper GI tract

The human gastrointestinal tract (GIT) is highly complex and on anatomical grounds, can be divided into five separate parts: mouth, oesophagus, stomach, small bowel and large intestine. The GIT plays host to a large range of bacteria, which help the body digest food, while boosting immunity to a range of antigens including microbes, from birth. The immune system requires the presence of commensal bacteria to facilitate its development and maintain its natural immunity to pathogenic microorganisms. These organisms are progressively acquired after birth and colonise from the mouth to the anus, both in luminal contents and over mucosal surfaces. Significant microbial growth occurs throughout this multi-compartment system, however, the density and complexity of individual microbiotas is defined by the structure and function of each organ. Microorganisms enter the mouth on inhalation and during eating. Many move through the GI tract to the stomach and lower gut, however, great numbers remain in the oral cavity. The mouth contains saliva consisting of enzymes such as amylase and lysozyme. The function of lysozyme is to destroy bacteria, however, these organisms often grow in biofilms on the tongue, cheek and teeth, which facilitates their protection from environmental hazards (Bowden and Hamilton, 1998). An estimated 750 different bacterial species may be present in the mouth, with numbers up to 10^6 cells per gram (Avila *et al.*, 2009). The mouth leads into the trachea of the upper respiratory tract, and the oesophagus of the digestive system. The oesophagus leads down to the stomach, with rapid movement of food via peristalsis and gravity. Little work has been done on colonisation of the oesophagus, although the few studies to date have indicated the presence of significant bacterial communities, with thirteen predominant genera at ca. 10^4 per mm^2 (Pei *et al.*, 2004; Macfarlane *et al.*, 2007). The stomach contains gastric acid

(pH <2), which is not suitable for bacterial growth (10^3 cells per gram), and acid tolerant bacteria such as streptococci and lactobacilli are most commonly found attached to the gastric mucosa (O'May *et al.*, 2005a). The small intestine has a rapid transit time for food (four to six hours), and this together with the secretion of pancreatic juices and acids reduces microbial colonisation. The distal ileum has little peristaltic movement compared to the duodenum, and contains less acid, allowing for increased bacterial colonisation, with bacterial numbers reaching 10^8 cells per ml (Macfarlane and Cummings, 1991). In contrast, the large intestine, which has a long transit time, facilitates bacterial colonisation and biofilm formation, with microbial cell densities reaching 10^{12} cells per gram of contents (Macfarlane *et al.*, 2000). Numerous microbiological studies have been done on the lower intestine, with great interest in inflammatory bowel diseases (IBD), such as Crohn's and ulcerative colitis (Macfarlane *et al.*, 2009).

The oesophagus is now becoming an area of greater interest due to the dramatic rise in incidence of BO and oesophageal adenocarcinoma over the past two decades. The microbiota and its dysregulation leads to chronic inflammation, as seen in gastric cancer and possibly colon cancer (Rowland, 2009). In the GIT, these organisms play a major role in immune regulation, and therefore, changes in bacterial composition in GORD due to cellular modifications may initiate and maintain neoplastic progression to oesophageal ADC.

1.3.1 Colonisation of the healthy oesophagus

The oral cavity plays host to a wide variety of bacteria, which are present in very high numbers. It is known that a change in the normal oral microbiota permits

colonisation by pathogenic bacteria, which can result in disease states such as gingivitis (Avila *et al.*, 2009). Unlike the oral cavity, there have been few studies on the microbiota of the healthy oesophagus, and therefore little consideration of the role bacteria may have in cancer of these tissues. The oesophagus can be infected with a number of organisms, including *Candida* (Dean and Burchard, 1998), *Cryptococcus*, mycobacteria (Jain *et al.*, 2002) and herpes virus (Nagri *et al.*, 2007), therefore, it is reasonable to assume that the healthy oesophagus may also play host to its own unique and commensal microbiota.

An initial study by Osias *et al.* (2004) carried out Gram stains and aerobic culture with biopsies from patients with a normal oesophagus. This was a highly elusive publication, omitting actual CFU counts, giving only Gram scores and percentages of patients with bacteria. However, results indicated colonisation occurred in the normal oesophagus (increasing with disease), with a majority of Gram positive cocci. A molecular based study by Pei *et al.* (2004) also examined microbial colonisation of the oesophagus, taking biopsies for PCR and cloning. This group only analysed biopsies from four patients that were known to be healthy, and although only one biopsy showed bacterial colonisation after culturing, all four had identifiable bacteria with PCR. Six phyla were identified among the four individuals: Firmicutes, Actinobacteria, Proteobacteria, TM7, Bacteroidetes and Fusobacteria. Genera included *Streptococcus*, *Veillonella*, *Clostridium*, *Rothia*, *Actinomyces*, *Bacteroides* and *Prevotella*, the majority of which appeared to be of oral origin. A culture-based study by Macfarlane *et al.* (2007) compared seven healthy and seven BO patient biopsies. Mucosal colonisation was found in three of the seven healthy individuals after aerobic, microaerophilic and anaerobic culture. Eighteen species

were identified in these patients from 16S rRNA gene sequencing including streptococci, lactobacilli, bifidobacteria, actinomyces, veillonella and prevotella.

These studies show that the oesophagus has its own distinct and diverse microbiota. Although the majority of species were of an oral origin, the oral phyla spirochaetes were not present, along with other commonly isolated oral organisms, providing evidence that not all oral bacteria can colonise these oesophageal tissues. However, larger studies are needed to confirm that the microbiota is stable over time, and common in all individuals.

1.4 Colonisation of the oesophagus in disease

1.4.1 Associations between bacteria and cancer

It has been estimated that 15.6 – 18% of cancers can be attributed to some form of infection (Pisani *et al*., 1997; Parkin, 2006). Infectious agents can cause a multitude of cancers: *H. pylori* (gastric cancer), human papilloma virus, hepatitis B and C, and Epstein-Barr virus (cervical, liver and Burkitts Lymphoma, respectively), the schistosome *S. haemotobium* (bladder cancer) and *Opisthorchis viverrini*, a liver fluke (gall bladder) (Parkin, 2006). Carriers of *Salmonella typhi* following enteric fever are at higher risk of developing gall bladder cancer (Kumar *et al.*, 2006). It is not yet understood how this species is involved in disease aetiology, however, the degradation of bile salts by these bacteria may produce substances which act as co-carcinogens (Aries *et al.*, 1969). In lung cancer patients, high levels of *Chlamydia pneumoniae* antibodies, specifically IgG and IgA, were seen (Koyi *et al.*, 2001). These high titres of IgA are indicative of chronic infection, and possibly chlamydia's role in the development of neoplasia. In 2005, Barry J. Marshall and Robin Warren were awarded the Nobel Prize for the discovery of *H. pylori* involvement in peptic

ulceration, gastric adenocarcinoma and gastric lymphoma (Marshall and Warren, 1983). Due to this discovery, it has been considered that bacteria may be a mechanism in driving the progression of BO and its associated adenocarcinoma.

1.4.2 Bacterial colonisation in GORD and BO

Two studies by Pei and co-workers at the New York School of Medicine have investigated bacterial colonisation in patients with reflux oesophagitis and BO (Pei *et al.*, 2005; Yang *et al.*, 2009). The first recruited 24 patients, 12 of which had GORD on histopathology examination. Broad range 16S rDNA gene PCR was employed for clone and sequence analysis, only two clones were analysed from each patient as part of this preliminary study. Seventeen species were identified in these patients, however, only nine were defined, with the other eight being unknown non-cultivated species. In contrast, only five species were detected in the BO patients. However, they then went on to analyse 99 clones from one of the BO patients, resulting in 21 bacterial species being identified. Greater than 60% of these species were classed as unidentified rumen and oral isolates, while 32% of species belonged to the genera *Prevotella*, *Gemella*, *Porphyromonas* and *Veillonella*. The second, much larger study from this group (Yang *et al.*, 2009) involved 34 patients (12 healthy, 12 GORD, 10 BO). Identical 16S rRNA gene analysis techniques were employed in the investigation, producing 200 sequences per sample. This study used hierarchical clustering analysis to investigate the genetic distance between sample microbiotas, and identified two distinct clusters; type I and type II microbiomes. The type I microbiome consisted mainly of aerobic streptococci (78%), with the other 22% being small percentages of other species (15% Gram negative). Conversely, the type II microbiota, which was predominantly found in oesophageal disease, had a

proportion of only 29% streptococci, with the majority being mainly *Gemella* spp. (8%) and Gram negative species (54%). The most common Gram negative species found in this group were anaerobic or microaerophilic, including prevotella/ bacteroides, neisseria, veillonella, haemophilus and fusobacteria. The type I microbiota was found in 11/12 normal patients, while the type II microbiota was found in 7/12 GORD and 6/10 BO patients.

The Macfarlane group studied 14 oesophageal samples from BO and normal patients equally (Macfarlane *et al.*, 2007). They obtained both aspirates and mucosal tissue for pH monitoring (aspirate only), and traditional culture of colonised bacteria for species identification with 16S rRNA gene sequence analysis. Bacterial populations and biofilm formation were increased in Barrett's patients compared with healthy subjects (Section 1.3.1). Although total CFU in healthy and BO patients were similar (ca. $\log_{10}$ 5.0 cells cm^{-2}), there was great species diversity (18 vs. 38 species) with only 10 common to both patient groups. Similar bacteria were found to those in other studies i.e. streptococci, staphylococci, gemella, veillonella, neisseria, prevotella and fusobacteria. Additionally, the potentially pathogenic bacterium *Campylobacter concisus* was found in 4/7 BO patients.

1.4.3 Colonisation in adenocarcinoma of the oesophagus

Studies have been published investigating the microbiota of the oesophagus in patients with carcinoma (Lau *et al.*, 1981, Finlay *et al.*, 1982, Mannell *et al.*, 1983) and megaoesophagus (Pajecki *et al.*, 2002). However, all of these studies used aspirate samples to culture aerobic and anaerobic bacteria, except Finlay's study, which analysed mucosal tissue. These investigations isolated mainly streptococci,

staphylococci and bacteroides.

There has been no work done on the presence of bacteria on oesophageal cells in adenocarcinoma patients, but with the presence of many different types of bacteria in healthy and Barrett's oesophageal tissue, it seems likely that a new and varied microbiota will be discovered. As oesophageal disease progresses, there is an increase in opportunistic pathogens, with a shift towards a Gram negative biofilm. It is likely that the microbiota in ADC patients will continue to become more diverse, and increasingly Gram negative due to the greater surface area of disorganised, morphologically varied, epithelial cells. The investigations in this study will aim to characterise the microbiota in patients with ADC.

Tables 1.2 and 1.3 summarise data from five key papers dealing with oesophageal microbiology, highlighting the main genera found, and the number of species in each genus identified. In the case of those using cloning methods, the number of clones sequenced are also noted.

Table 1.2: *A summary of the main publications relating to the oesophageal microbiota in health and disease.*

Methodology	Demographics	Results	Reference
Histology, culture and Gram staining	65 patients (BO HGD, BO LGD, BO, GORD, control), age 31-87	Gram positive cocci (37/47), histological bacterial score BO > non-BO, increasing through disease. Bacteriological scores PPI > non-PPI.	Osias *et al*., 2004
16S rDNA clone libraries	4 healthy patients (veterans), age 49-79	6 bacterial phyla collectively (Firmicutes, Actinobacteria, Proteobacteria, TM7, Bacteroidetes, and Fusobacteria)	Pei *et al*., 2004
16S rDNA clone libraries	24 patients (GORD, BO and control), age 34-81	17 species identified throughout, followed by full analysis of one BO patient (21 spp. incl. Prevotella, Gemella, Veillonella and Porphyromonas).	Pei *et al*., 2005
Fluorescent *In Situ* Hybridisation and culture	14 patients (BO and control), age 36-88	18 (non-BO) vs. 38 (BO) species, with increased biofilm formation. *Campylobacter concisus* also found in 57% BO patients.	Macfarlane *et al*., 2007
16S rDNA clone libraries, & microbiome cluster analysis	34 patients (GORD, BO, control), age 34-81	Two microbiome types (I & II). Type I 78% Streptococcus, Type II 29% Streptococcus. Controls 11/12, GORD 5/12, BO 4/10 type I microbiome.	Yang *et al*., 2009

Table 1.3: *Summary of the main oesophageal species found in key research articles.*

Phylum	***Genus***	**Pei *et al.*, 2004** [a]	**Pei *et al.*, 2005** [b]	**Macfarlane *et al.*, 2007** [c]	**Yang *et al., 2009*** [d]
Firmicutes	*Streptococcus*	4 (9, 374)	2, 2, 1 (2)	3, 2 (3, 6)	13, 13, 13
	Staphylococcus	ND [e]	ND	0, 1 (2)	ND
	Peptostreptococcus	1 (1, 2)	ND	ND	2, 2, 1
	Megasphaera	4 (1, 19)	ND	0, 2 (2)	ND
	Gemella	4 (3, 14)	0, 0, 1 (1)	0, 1 (1)	3, 3, 3
	Veillonella	4 (3, 131)	1, 0, 1 (3)	1, 2 (1, 2)	3, 4, 5
	Lactobacillus	2 (5, 26)	ND	1, 0 (2)	3, 0, 6
	Clostridium	4 (7, 27)	ND	ND	0, 1, 0
	Selenomonas	2 (4, 5)	ND	ND	ND
Bacteroidetes	*Bacteroides*	4 (2, 15)	ND	ND	1, 2, 3
	Prevotella	4 (17, 157)	5, 2, 1 (4)	2, 2 (2, 3)	17, 13, 12
	Porphyromonas	2 (3, 9)	0, 0, 1 (1)	ND	2, 4, 1
	Capnocytophaga	ND	ND	ND	2, 2, 2
Actinobacteria	*Actinomyces*	4 (4, 12)	ND	2, 2 (2, 2)	1, 3, 2
	Rothia	4 (2, 22)	0, 0, 1 (1)	ND	2, 1, 2
	Bifidobacterium	ND	ND	1, 0 (1)	1, 0, 0
	Propionibacterium	ND	ND	1, 0 (1)	ND
	Corynebacterium	ND	ND	ND	0, 1, 3
Proteobacteria	*Neisseria*	2 (3, 3)	0, 0, 1 (1)	0, 1 (1)	6, 3, 2
	Campylobacter	2 (2, 4)	1, 0, 1 (1)	0, 4 (2)	2, 2, 1
	Helicobacter	ND	0, 3, 0 (1)	ND	ND
	Haemophilus	4 (4, 7)	0, 2, 0 (2)	ND	6, 6, 5
Fusobacteria	*Fusobacterium*	3 (4, 10)	ND	0, 1 (1)	3, 2, 2
	Leptotrichia	2 (2, 10)	ND	ND	2, 4, 4
TM7	*TM7*	4 (3, 13)	ND	ND	3, 3, 2

[a] No. of patients positive for genus in 4 control patients (no. of species, clones). [b] No. of clones per genus: 9 control, 12 GORD, 3 BO (no. of species). [c] No. of patients positive for genus: 7 control and 7 BO patients (no. of species). [d] No. of species for each genus: 12 control, 12 GORD, 10 BO

patients. [e] *ND, not detected.*

1.5 The microbiota

1.5.1 Oral biofilms

As with much of the human microbiota, microbiological analysis of diseased tissue has been more extensively studied than healthy. The oral microbiota is no exception, with many research articles on the oral biofilm of patients with periodontal diseases. Cultivation of bacteria from the oral cavity is problematic, with more than 50% being difficult to culture. However, over 700 bacterial species have been identified (Aas *et al.*, 2005). Molecular cloning techniques have produced over 2,500 16S rRNA clones, yielding 141 different taxa. These included *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria* and TM7 (never cultivated). The main genera found were streptococci, gemella, veillonella, actinomyces, neisseria and rothia. However, fusobacteria, porphyromonas, campylobacter and leptotrichia were also found.

1.5.2 Biofilm interactions

A biofilm is a population of individual or multiple species, which can develop from a single species or community of cells. The biofilm represents an interdependent community, and can grow on a variety of biotic or abiotic surfaces. Some bacteria present in biofilms will produce extracellular products (EPS, nucleic acids, proteins) which provide essential nutrients, growth components and protection for the community. A biofilm population can contain various groupings of bacterial types called guilds, which have an array of metabolic requirements (Davey and O'Toole, 2000). Varying guilds include fermenters, methanogens, sulphate- and sulphur-reducers. Chemical connections in these biofilms allow communication between

bacteria, promoting survival strategies; this is named quorum sensing (Shapiro, 1998). Figure 1.8 shows the development of a biofilm from its early establishment to maturity.

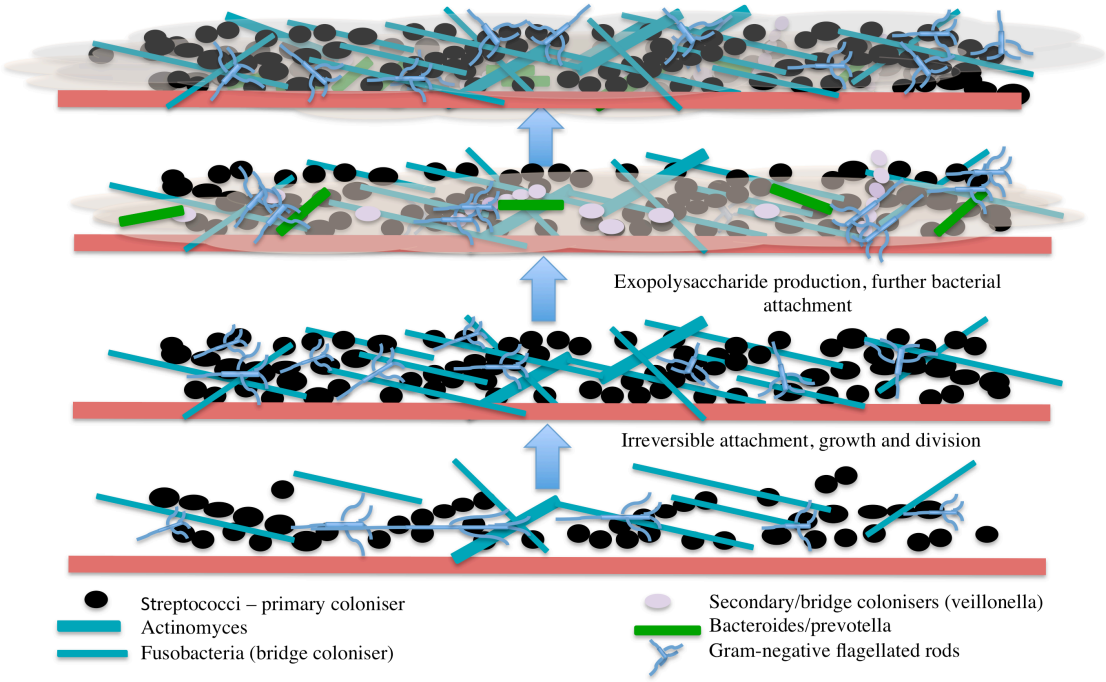


Fig. 1.8: *Diagram detailing the development of a biofilm. Early colonisers such as streptococci, actinomyces and fusobacteria (a bridge organism) associate with epithelium. This is followed by attachment, accumulation, exopolysaccharide production and further bacterial attachment.*

Streptococcal species are main organisms found in the healthy oesophagus and oral cavity. In the mouth, they are the primary (early) colonisers, along with Gram positive rods such as *Actinomyces naeslundii*. This genus is mostly commensal, although *S. mutans* is associated with dental caries. In the studies mentioned previously, the principal bacteria found in the oesophagus were streptococci, actinomyces, veillonella, fusobacteria and prevotella/ bacteroides. Veillonella (*V. atypica* and *V. parvula*) is a secondary (late) coloniser, being able to coaggregate with streptococcus. Veillonella can also coaggregate with fusobacteria (*F. nucleatum*), which is classed as a bridge organism due to its ability to adhere to both primary and secondary colonisers. Fusobacteria can also attach to actinomyces,

porphyromonas, *H. pylori*, and directly with streptococci (Rickard *et al.*, 2003; Kreth *et al.*, 2009). Streptococci are major colonisers of the oesophagus where they adhere to epithelial cells directly. Therefore, they have close interactions with the immune system, and can mediate the effects of other species when coaggregated. For example, *F. nucleatum* triggers expression of IL-8, however, when adhered with streptococci, the effect is attenuated (Kreth *et al.*, 2009). Biofilms have been described as a "consortia of cells that often possess a metabolic activity greater than that of the component species" (Rickard *et al.*, 2003). They must be able to withstand stresses due to shearing, washing and anti-microbial treatments. Therefore, in the oesophagus, it is likely that bacteria grow in biofilms (Macfarlane *et al.*, 2007) to withstand the harsh environment.

1.5.3 Helicobacter pylori

As discussed previously in this chapter, *Helicobacter pylori* are the main aetiologic agent in gastric cancer development, being able to induce gastritis after its introduction to previously negative patients. There has also been much debate over the role of *H. pylori* in oesophageal ADC development. The majority of studies concur that this bacterium has a protective effect against this form of cancer, and that there is an inverse relationship with BO and ADC (de Martel *et al.*, 2005). Large community-based studies by Anderson *et al.* (2008) and Corley *et al.* (2008) investigated a total of 1870 patients (542 GORD, 542 BO, 227 ADC and 559 controls). Serological antibody testing with western blots gave data on *H. pylori* and CagA status, while enzyme assays were used to measure gastric atrophy, as defined by pepsinogen I/II ratios. These two studies confirmed the inverse association between *H. pylori* and oesophageal cancer: CagA seropositivity was lower in

oesophageal compared with junctional adenocarcinomas, and there was a reduced risk of GORD, BO and ADC in patients with the seropositive bacterium. Approximately nine percent of patients with the *H. pylori*-seropositive phenotype had gastric atrophy compared with 1.4% of seronegative patients. This latter result suggests that reduced oesophageal adenocarcinoma is not simply due to gastric atrophy and that other mechanisms are involved. Other mechanisms of action have been proposed for this negative association, including the presence of *H. pylori* at the gastric cardia leading to atrophy of acid secreting mucosa, or the production of alkaline ammonia substances protecting the oesophageal mucosa from acid (McColl *et al.*, 2008).

1.5.4 Campylobacter species

Campylobacters are so named for their twisted shape; they are spiral, curved slim rods, with a Gram negative morphology (Fig. 1.9). These bacteria have a single or bi-polar flagellum allowing rapid movement. Campylobacters prefer a microaerophilic environment, however, a number of species can survive anaerobically. The cell wall of these organisms is typically Gram negative, and with the ability to shift their antigenic composition, campylobacters can change the LPS molecules involved in adherence. The natural habitat for campylobacters is the intestines of warm-blooded animals, particularly birds and poultry. They are also found in the oral cavity and urogenital tract of humans and during infections of the gut. Human infection is thought to occur through the faecal-oral route, due to food contamination, contaminated water and handling of livestock (Bourke *et al.*, 1998; Miller and Mandrell, 2004).

Fig. 1.9: *Scanning electron micrograph of Campylobacter jejuni. Photo by De Wood; digital colorization by Chris Pooley, USDA.*

Although campylobacters were identified over 50 years ago, our understanding of their mode of pathogenesis is still relatively limited. These bacteria are not easily cultured, having variable biochemical activities and a time consuming, fastidious growth pattern. Due to their highly similar molecular sequences, campylobacters are also not easily identified with standard molecular techniques. *Campylobacter jejuni* was the first food-borne pathogen to be completely sequenced (Parkhill *et al.*, 2000). This group found many regions of hypervariability, specifically in regions encoding biosynthesis and the modification of surface structures. Major structural changes can occur in these bacteria due to insertion of new DNA sequences, and this could account for their varied behaviours and modes of interaction with the human host.

Taxonomy, classification and origins of campylobacters

Members of the genus *Campylobacter* were first identified and classified as vibrios due to their spiral appearance, however, in 1963 they were reclassified forming a new genus (Sebald and Veron, 1963). After many years of taxonomic and structural

analyses, these bacteria are now part of the *Campylobacteraceae* family (Vandamme *et al.*, 1990); which also comprises the genera *Arcobacter* and *Helicobacter*. As with the sequencing of many Proteobacteria, polyphasic rRNA homology and DNA-DNA hybridisation experiments were carried out to complete their phylogenic tree. This amended genus now contains seventeen species, six subspecies and two biovars (bv): *C. coli*, *C. concisus*, *C. curvus*, *C. fetus* subsp. (*fetus* and *venerealis)*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis* subsp. (*hyalointestinalis* and *lawnsonii)*, *C. insulaenigrae*, *C. jejuni* subsp. (*jejuni* and *doylei*), *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum* (bv. *faecalis* and *paraureolyticus*), and *C. upsaliensis* (Korczak *et al*., 2006). Of these species, only 15 have been shown to cause human illness. *Campylobacter helveticus* has only been found in cats and dogs, while *C. hyalointestinalis* subsp. *lawsonii* is found in the stomachs of pigs with ulcerative disease. Although previously identified in human disease, infections with *C. mucosalis* are now thought to be misdiagnosed cases of the closely related *C. concisus* (On, 1994).

Campylobacteriosis in humans

Infections with campylobacters are now three times as common as those with salmonella. The Center for Disease Control reports that approximately 2.4 million persons are infected every year with *Campylobacter*, and these occur more frequently in the summer months, with an estimated 124 persons dying of this infection annually. Human intestinal disorders are most commonly caused by *C. jejuni* and *C. coli* (Skirrow, 1994). *Campylobacter fetus* and *C. jejuni* can inititate pericarditis and myocarditis (Uzoigwe, 2005), while *C. rectus* is found in the oral cavity of patients with periodontal infections (Ihara, 2003). *Campylobacter jejuni* is

also associated with the autoimmune disease Guillain-Barre Syndrome (GBS) (Kuwabara, 2004), an acute peripheral neuropathy with shredding of the nerves along the spine leading to muscle weakening and paralysis of the limbs and face. This disease can also be triggered by the Epstein-Barr virus, cytomegalovirus and *Mycoplasma pneumoniae*. *Campylobacter jejuni* infection leads to the acute motor axonal neuropathy (ANAN) subtype of GBS, considered to be a result of molecular mimicry. Reports of the AIDP (acute inflammatory demyelinating polyneuropathy) form of GBS being due to *C. jejuni* infection were recently invalidated by a patient study (Kuwabara *et al.*, 2004). A subsequent post-campylobacter autoimmune disease is reactive arthritis (ReA). Patient studies in Oregon and Helsinki have shown that 88% and 10% of patients with this syndrome previously had either *C. jejuni* or *C. coli* infection, respectively (Hannu *et al.*, 2002; Townes *et al.*, 2008). With campylobacters being involved in a number of human infections, potentially resulting in more serious conditions, it is important that their mode of pathogenicity is clearly understood, allowing successful identification and treatment of these patients.

1.5.5 Mechanisms of virulence in campylobacter

Campylobacters possess a number of virulence factors that contribute to their survival inside the host, and subsequent progression to disease. The ability of these organisms to move rapidly using their flagella allows chemotaxis and invasion of host epithelial cells. It has recently been shown that these flagella not only facilitate the above functions, but that they are also involved in the secretion of virulence proteins, and allow microcolony formation and avoidance of host innate immune responses (Guerry, 2007).

Flagella

Flagella are constructed of a basal body, filament and hook. A motor in the bacterial cell envelope drives rotation of the filament, with the hook acting as a coupling structure between filament and motor (Bardy *et al.,* 2003). Flagella are an important virulence determinant for bacteria, and are essential to the majority of gastrointestinal disease causing organisms, including salmonella and other enterobacterial species.

Studies with *C. concisus* isolated from subgingival plaque showed that this species demonstrated chemotactic movement towards formate (Paster and Gibbons, 1986), whereas with *C. jejuni*, mucin was the strongest chemoattractant (Hughdahl *et al.*, 1988). Hughdahl investigated the ability of individual mucin constituents to chemoattract these organisms, and found that intermediates of the TCA cycle were the main source of chemotaxis. Similar findings were observed with *S. typhimurium*, *E. coli*, *V. cholerae* and *P. aeruginosa* (Allweiss *et al.*, 1977). Campylobacters use intermediates of the TCA cycle as energy sources, with L-fucose being the predominant stimulus, operating through upregulation of *Fla*A promoter regions (Allen and Griffiths, 2001).

Campylobacter invasion antigens (Cia proteins)

Interaction and invasion of human intestinal cells has been modelled with a number of gut epithelial cell lines (Fauchere *et al.*, 1986). More recently, Grant *et al.* (1993) have investigated the role of campylobacter virulence factors in their internalisation and translocation across the epithelial cell barrier. These bacteria adhere to the epithelial surface using their flagella, LPS coating and various secreted proteins.

This research group constructed flagella mutants that were unable to cross the epithelial barrier, indicating that either complete motility or an active *flaA* gene is needed for this action. Malik-Kale *et al.* (2008) grew *C. jejuni* on agar plates with the bile acid sodium deoxycholate. Under bile acid stress, the bacteria had amplified expression of virulence genes, including *ciaB*, and increased secretion of Cia proteins, although there was no change in motility.

Campylobacter survival strategies

Campylobacters can survive in a number of mammalian cell types. During gastrointestinal infection, they reside within epithelial cells, macrophages and paracellular structures of the lamina propria. However, these organisms can produce a number of toxic species, such as hydrogen peroxide, during aerobic metabolism, which can then form more toxic intermediates when in contact with nitric oxide sythase and reduced iron products. These toxic substances such as hydroxyl radicals and nitrogen dioxide aid survival. Catalase formation by these organisms provides resistance to hydrogen peroxide through its inactivation, therefore increasing intra-macrophage survival (Day *et al.*, 2000). A further study by Pogacar *et al*. (2009) used murine macrophage cell lines to study *C. jejuni* infection and survival under normal or stressed conditions. After environmental stress was induced (oxygen, heat or nutrient starvation), survival was impaired, however, regardless of stress it was reported that these bacteria could not survive for more than 30 hours inside this hostile cell. Nutrient limitation reduced the ability to adhere to and invade mammalian cells, however, oxidative stress for five hours increased their adhesive and invasive properties. This could be due to upregulation of mechanisms that inhibit toxic oxygen products, therefore enhancing their persistence in the host.

Cytolethal distending toxin (CDT)

CDT was identified over 20 years ago (Johnson and Lior, 1987) when Chinese Hamster Ovary (CHO) cell lines infected with *E. coli* became distended and died. This group went on to look at its presence in other gastrointestinal pathogens, leading to its discovery in campylobacters (Johnson and Lior, 1988). CDT has now been purified and sequenced from a number of pathogenic bacteria, including *Shigella* and *Campylobacter*. To date, the protein has been identified in *C. jejuni*, *C. coli*, *C. lari* and *C. fetus*, and a recent study found a CDT-like effect on Vero cells by *C. concisus* (Engberg *et al.*, 2005). This toxin is the product of three genes *cdt*A, *cdt*B and *cdt*C, where *cdt*AC are binding units for *cdt*B that allow its entry into a host cell. Once inside the cell, *cdt*B enters the nucleus producing a DN*ase* I-like activity leading to double strand breaks in host DNA. This damage results in a block at the G2 phase of the cell cycle prior to mitosis, the cells can no longer divide, become distended and die (Smith and Bayles, 2006). If there are mutations in any genes of the *cdt*ABC complex, cytotoxicity is lost.

Surface lipopolysaccharide

LPS is a surface structure found anchored in the outer membrane of Gram negative bacteria. This molecule contains three parts; the lipid A moiety found in the outer membrane, the core and O-antigen. Lipid A is endotoxic, being released during bacterial killing, resulting in an inflammatory response. Bacterial LPS is involved in adherence to host cells, and the component molecules on the surface can vary in their antigenic profile, enabling immune avoidance. The core of this structure is built up of various sugars, and the presence of sialic acid has been associated with GBS when attached to β-D-galactosidase (Schwerer *et al.*, 1995). A recent patient study by

Mortensen *et al.* (2009) further investigated the role of sialylation in *C. jejuni* in association with disease severity and joint pain/reactive arthritis. Campylobacters with a sialic acid positive lipo-oligosaccharide (LOS) isolated from patients were correlated with bloody diarrhoea, increased duration of illness and arthritic symptoms. LPS and its components are very important factors in campylobacter infection, where sialylation increases serum resistance, therefore prolonging their presence in the host. Additionally, these molecules may increase bacterial invasion abilities and the host inflammatory response.

Nitrate reduction and nitric oxide (NO)

Many campylobacters are nitrate reducers, being able to produce nitrite via the enzyme nitrate reductase (Pitmann and Kelly, 2005). Nitrite can then be reduced to NO in the presence of acid. As mentioned earlier, NO is an antimicrobial agent, that modifies bacterial DNA and respiratory complexes. Interestingly, humans do not possess nitrate reductase, relying on oral bacteria to reduce salivary nitrate to nitrite, thereby implying a symbiotic relationship. One patient study found that individuals with fewer dental caries had increased concentrations of salivary nitrate and a microbiota with enhanced nitrate reducing ability (Doel *et al.*, 2004). This group conducted a further study investigating the nitrate-reducing bacteria present in the oral microbiota (2005). The main species found were *Veillonella*, *Actinomyces* and *Rothia*, with the tongue containing the largest nitrate reducing potential of mainly Gram negative cocci (58.1%).

Once oral bacteria have reduced salivary nitrate to nitrite, it is swallowed and encounters gastric acid, whence it is converted to NO. In a patient-based study with

15 healthy *H. pylori*-negative volunteers, pH, NO concentration, serum nitrite and salivary nitrate were measured at the OGJ. After ingestion of a nitrate meal, pH and NO levels were significantly higher at the OGJ and gastric cardia compared with areas above and below this region (Iijima *et al.*, 2002). Suzuki *et al*. (2005) progressed this work, investigating the effects of a nitrate meal in 10 BO patients. Simultaneous recordings of pH and nitric oxide concentration were made throughout the oesophagus before and after nitrate consumption. Before feeding, reflux episodes did not increase NO, however, after nitrate ingestion reflux episodes reducing pH to 1-2 generated 20 μM nitric oxide. This can have a number of physiological effects in the body, and could possibly lead to mutagenesis and carcinogenesis in this part of the gut (Burnett *et al.*, 2000). If NO is further oxidised to N_2O_3, deamination can occur through reaction with primary amines on DNA bases (Burney *et al.*, 1999). Production of N-nitrosocompounds via NO reactions with secondary amines can also result in nitrosative damage of DNA (Wink *et al.*, 1999).

Previous studies of the oesophageal microbiota have identified the presence of nitrate reducing *Veillonella*, *Rothia* and *Actinomyces* on the oesophageal mucosa. Therefore, in patients with reflux disease, where acid is introduced regularly, NO production could lead to inflammation and damage. Additionally, Macfarlane *et al.* (2007) found the pathogenic nitrate-reducing species *Campylobacter concisus* in patients with reflux disease. Campylobacters could therefore be involved in the initiation and/or maintenance of carcinogenesis through NO formation and the subsequent inflammatory response.

1.5.6 Immune response to campylobacters

Campylobacter infection initiates an innate immune response in the host, through the activation of TLR. Wang *et al.* (2000) investigated the inflammatory effect of *C. rectus* on Hep-2 cells (larynx) over a period of up to three days, to examine the role of these organisms in periodontitis. Production of IL-6, IL-8 and TNF-α was significantly increased in the human cell line, with a transient increase in IL-1α and IL-1β at 1-2 hours post infection. *Campylobacter jejuni* is the most common test species in this genus, because it is the most commonly found agent in gastroenteritis. Therefore, the majority of studies use this organism in modelling experiments. Hu *et al.* (2006) infected dendritic cell (DC) cultures with *C. jejuni* to monitor the inflammatory response. DC are antigen-presenting cells (APC), they are part of the innate and adaptive immune response, and play a key role in the initial reaction to microbial infection. This study found that these bacteria entered the cells within two hours, and up-regulated CD40, CD80 and CD86 surface components, a sign of cell maturation. These infected DC also triggered an innate and Th1 response, represented by IL-1β, IL-6, IL-8, IL-10, TNF-α, IL-12 and IFNγ. Zheng *et al.* (2008) then investigated the role of *C. jejuni*'s CDT on intestinal epithelial cells. Bacteria were added to T84 monolayers at a MOI of 10. Eleven different strains showed varying degrees of effectiveness with respect to adhesion, invasion and transcytosis (movement within cell). Despite these variations, all strains induced IL-8 expression, with similar levels for cell-free supernatants compared with live culture. The group also produced CDT and flagellar mutants, which invoked a lower IL-8 response, indicating the importance of these for the innate immune response through TLR/NF-kappa B signalling.

A recent study by Friis *et al*. (2009) measured the IL-6 response of Caco-2 cells to *C. jejuni* (MOI of 1000) compared with cells mutated for TLR-2 and MyD88 (part of NF-kappa B signalling pathway). MyD88-mutant cells had similar IL-6 expression to normal Caco-2 cells, whereas TLR-2 mutants had reduced levels of secretion on ELISA testing after three hours. Stimulation with purified surface polysaccharides (capsular polysaccharide) showed a correlation between concentration of polysaccharide and IL-6 secretion.

These studies indicate that campylobacters induce an innate response through dendritic cells of the immune system and direct contact with epithelial cells. Production of IL-1β, IL-6, IL-8, TNF-α and IL-12 through TLR related NF-kappa B signalling mirrors the immune response seen in *H. pylori* infection of the gastric epithelium resulting in chronic inflammation and metaplasia.

1.6 Summary and research hypothesis

Summary

- BO is a progressive condition resulting from acid/bile refluxate;
- This condition is multi-factorial, involving acid/bile, HH, obesity, gender, ethnicity, diet, genetics and the resulting inflammatory response;
- Genetic and epigenetic modifications are key markers of oesophageal ADC;
- Epithelial damage as a consequence of refluxate leads to sensitive cells, which are more prone to continuing damage;
- BO hosts an increasingly Gram negative microbiota compared with the squamous cell oesophagus;

- Gram negative bacteria have increased LPS, bacterial flagella and ability for N-nitrosocompound formation;
- LPS induce TLR/ NF-kappa B pathways;
- NF-kappa B pathways increase production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6;
- The presence of bacteria in the distal oesophagus can lead to increased production of ROS and nitrosative compounds when presented with acid;
- ROS and NO trigger genetic modifications and inflammatory responses (cytokines, COX-2).

The continual presence of acid and bile salts in the oesophagus due to reflux disease results in sensitive epithelial cells. Dietary and salivary nitrates are converted to nitrite by oral bacteria, and possibly, the oesophageal microbiota when swallowed. Nitrite is normally converted to NO in the stomach, when in contact with gastric acid, however, in patients with acid reflux, NO can be produced in the distal oesophagus during a reflux episode. This process further damages the epithelium leading to an inflammatory response.

Hypothesis

The research hypothesis is that microbial composition in the distal oesophagus changes throughout disease, becoming increasingly Gram negative in adenocarcinoma patients. Through a variety of mechanisms, these bacteria may initiate or maintain this chronic inflammatory condition, with a possible key involvement for nitrate-reducing *Campylobacter* species. Comparisons between *H. pylori* involvement in gastric adenocarcinoma and the progression of BO to

oesophageal ADC have been made in this Introduction. Many comparable events take place between these two neoplastic pathways, with almost identical immune responses and genetic changes. Therefore, campylobacter, a helicobacter-like organism may be a key aetiologic agent in this disease.

Aims of this study

This project aims to recruit statistically significant cohorts of control, GORD, BO and ADC patients for real-time PCR analysis of major oesophageal genera. These main bacterial groups will be identified by cultural analysis of a small subset of each patient cohort. Once key organisms and population changes have been identified, *in vitro* models will be designed to further investigate biofilm composition, and the effects of refluxate on virulence expression. These models will be in the form of continuous culture (chemostats), and oesophageal cell lines. The aim will be to identify key changes in inflammatory responses and DNA modifications, proving the importance of bacteria in the development of oesophageal adenocarcinoma.

Chapter 2

Bacterial colonisation of the oesophagus in different stages of disease

2.1 Introduction

The study of microbiology goes back three centuries, and although modern technologies allow for the identification of medically related microorganisms genetically, they should not be viewed as being able to completely replace the culture of viable bacteria. Microorganisms are constantly introduced to the human body, and opportunistic bacteria adhere to epithelia and biofilms if the environment is suitable and nutrients are available. Therefore, when looking for an aetiologic agent in gastrointestinal diseases, the viability of bacteria colonising epithelial surfaces should be fully investigated.

For these reasons, the work described in this thesis has employed traditional culturing methods to identify the principal viable microorganisms colonising inflamed tissues in groups of patients with oesophageal disease; the resulting data facilitating the development of standards for real-time PCR detection and quantification of the target organisms in larger patient cohorts. The use of traditional culturing methods has allowed development of *in vitro* models, using clinical isolates with cell lines and in continuous culture. These experiments were designed to investigate the impact of refluxate on the virulence of the oesophageal microbiota, and the consequent oncogenic effects in epithelial cells.

2.1.1 Molecular tools in microbiology

It is true that without molecular technologies, which are mainly based on 16S rRNA approaches, knowledge of the human gastrointestinal microbiota would still be very limited. These methodologies, such as PCR/sequencing, dot-blot hybridisation, microarrays, metagenomics (clone libraries), fluorescent *in situ* hybridisation (FISH)

and real-time PCR can be time-effective, more sensitive and most importantly allow for identification of non-culturable bacteria (Zoetendal *et al.*, 2006). However, without the ability to culture these bacteria originally for identification, DNA and gene sequences, phenotypic and metabolic data, would not be available. Many of these techniques require primers or a probe, which relies on effective sequencing of these organisms. There is a large variety of primer sequences in the literature for many intestinal bacteria, however, with genetic diversity between each species and sub-species, finding primers that are universal for each genus and species can be challenging. The majority of research with *Campylobacter* species use molecular techniques, however this study has found that many of the primer sets do not work on isolates from different niches. Therefore, it is extremely important that the art of traditional culture is not lost completely to molecular approaches, and that the two can be used in harmony.

2.1.2 Microbial Identification System (MIDI)

A number of methods are available for identifying intestinal bacteria, with both culture and chemotaxonomy and the more modern molecular methods such as PCR and clone libraries. However, sequencing involved in some types of molecular analysis is expensive when all of the cultured bacteria from 34 patient biopsies are to be identified, as in this study. It was therefore necessary to find a less expensive, yet fast and reliable method for analysing all of the clinical isolates obtained in this research.

All bacteria possess fatty acids, not only in cell membranes, but also in LPS and lipoteichoic acids, and over 300 different types have been found to date. Each

bacterial genus and species has its own specific composition of between 5 and 10 cellular fatty acids (CFA), analogous to a human fingerprint. Each fatty acid has between 9 and 20 carbon atoms on average, and the length, branching groups and bonding positions vary between each genus and species dependent on their biosynthetic pathways. These changes in length (odd or even carbon atoms), saturation and branching vary among Gram negative and Gram positive organisms, with some CFA being common to all, such as hexadecanoic acid (palmitoleic acid), which is found in most prokaryotes (Welch, 1991). When these fatty acids undergo catalysis with methanol, they produce fatty acid methyl esters (FAME). The MIDI system uses gas chromatography (GC) to analyse these extracted cellular fatty acids from individual isolates. The system contains a number of libraries (aerobic, anaerobic, clinical), which allow comparisons of unknown fatty acid profiles with those of known bacteria, using dendograms, for fast identification through pattern recognition. Cellular fatty acids from bacterial lipid membranes can be affected by environmental changes, therefore, culturing for FAME extraction must be carried out under precise conditions to prevent changes to their relative proportions. The media used and nutritional content, incubation conditions, and injection port temperatures must be uniform throughout all analysis.

The MIDI system has a library of over 100,000 FAME profiles for bacterial genera, species and strains collected worldwide (Stager and Davis, 1992). The ability of this technology to correctly identify Gram negative species is 80-93% (Olson, 1996), and compared with other chemotaxonomic systems, which use substrate utilization for recognition, its ability to identify to the species and strain level is more reliable.

2.1.3 The oral and gastric microbiotas

As described previously in Section 1.5.1, a small study of the healthy oral cavity found 141 taxa, belonging to six phyla, isolated from nine areas of the mouth (Aas *et al.*, 2005). The study used PCR amplification of 16S rRNA genes to clone and sequence the samples. Streptococci were the main genus found, with *S. mitis* being found in all locations, comprising >15% of the total number of clones in the majority of patients and sites. The opportunistic pathogen *Granulicatella adiacens* was also found in all sites in the five patients examined. Prevotellas colonised all oral surfaces investigated, while *Rothia denticariosa* was only found on teeth and subgingival surfaces. Campylobacters were found in four of the five patients, with *C. concisus* being the main species found, however, *C. gracilis, C. showae* and *C. curvus* were isolated from tonsil samples and subgingival plaque (Aas *et al.*, 2005).

Studies into oral diseases identify *Porphyromonas gingivalis*, *Treponema denticola* and *T. forsythia* as the main aetiologic agents in gingivitis and periodontitis. These species were not found in any healthy patients, however, streptococci (*S. mitis*, *S. oralis*, *S. angiosus*), rothia, actinomyces, lactobacilli, bifidobacteria and candidas have been shown to be part of these cariogenic plaques (Filoche *et al.*, 2010; Kumar *et al.*, 2003); many of which were also not found as part of the healthy oral microbiota. This data indicates that these oral diseases are polymicrobial rather than being due to a single species or genus. Bacteria colonising oral sites should be looked at as a microbial ecosystem, where all of the microorganisms in the niche function together, in the context of both biotic and abiotc factors (Filoche *et al.*, 2010). Bacteria in oral biofilms survive in the community due to interdependent relationships, both metabolically and ecologically, and this may explain why so

many of these organisms cannot be cultured in the laboratory, and have only been detected using molecular techniques.

In the case of the gastric microbiota, only a small number of species can survive due to the harsh environment. The main organisms found are streptococci and lactobacilli, and in a number of individuals, *H. pylori*, with total eubacterial numbers reaching 10^3 CFU ml^{-1} (O'May *et al.*, 2005a). The low pH found in the stomach suppresses colonisation of most microorganisms, with a pH <4 being the main barrier to microbial growth. However, biofilms can create protective niches, allowing organisms that would not normally survive to co-exist with acid tolerant species such as lactobacilli, thus providing metabolic synergy (Stoodley *et al.*, 2002). Many patients with *H. pylori* do not develop gastric disease, however, it can lead to gastric cancer in those who develop corpus gastritis. This condition leads to hypochlorhydria, where production of gastric acid is reduced, resulting in increased pH, and therefore, enhanced microbial colonisation (Dicksved *et al.*, 2009). This study used terminal restriction fragment length polymorphism (T-RFLP) techniques to clone and sequence gastric biopsies from 10 cancer and 5 control patients. Five phyla were identified (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria) in the gastric cancer group. The abundance of *H. pylori* was very low, being found in only one of the cancer patients. This research corresponds with previous reports of hypochlorhydria, indicating that although *H. pylori* creates an altered environment for pathogenesis, it is the developing community as a whole which progresses the disease to cancer, possibly via the production of N-nitrosocompounds.

The normal oesophageal microbiota is believed to originate from the oral cavity, with a proportion of the organisms found in the oesophagus being part of the normal microbiota in the mouth. In patients with GORD, BO and ADC refluxate from the stomach may also contain bacteria able to colonise the oesophageal mucosa, resulting in a newly developed and unique microbiota.

This study aims to identify the predominant organisms colonising the oesophagus, and to investigate whether there are specific species present in diseased patients which promote pathogenesis, or whether a complete shift in the microbial community promotes this reaction.

2.2 Materials and Methods

2.2.1 Patient recruitment

Tissue for analysis of biofilm composition, structure and related host responses was obtained from patients during attendance for an upper gastrointestinal endoscopy at Ninewells Hospital Endoscopy Unit. Patients with gastro-oesophageal reflux disease (37), Barrett's oesophagus (45), oesophageal adenocarcinoma or squamous cell carcinoma (34), and normal controls (39) were recruited. Patients were all aged >18 years, with controls attending for upper GI endoscopy that was not related to oesophageal illness. Patients were excluded if they were receiving any medications that could disrupt their normal microbiota, principally antibiotics.

Four biopsies were taken from each patient approximately 5 cm above the oesophagogastric junction (OGJ), at place of inflammation, BO or ADC site. Eight biopsies from each cohort (10 from ADC) were analysed for bacterial colonisation

and total cell counts, while all others were frozen (-80^{o}C) for subsequent real-time PCR analysis (bacteria and cytokines, Chapter 3).

The patients gave informed consent for participation in the investigation, and the Tayside Committee on Medical Research Ethics approved the study.

2.2.2 Traditional culturing of oesophageal biopsies

Eight specimens from the control, GORD and Barrett's cohorts, and 10 from the cancer patients were cultured on a variety of growth media. Upon collection, the biopsies were placed in Wilkins-Chalgren broth for carriage to the laboratory for immediate processing. Individual biopsies were homogenised with half strength peptone water, and serially diluted to 10^{-3} before spreading on a wide range of selective plates in duplicate. Selective culture media used were as follows: Nutrient agar; MacConkey agar No. 2 (both aerobic); Blood azide agar (aerobic and anaerobic); Columbia blood agar (with *Campylobacter* and *Helicobacter pylori* growth supplements); Chocolate agar (all microaerophilic growth); Columbia blood agar supplemented with succinate, nitrate, formate and fumarate (1g L^{-1} each, Sigma-Aldrich, UK) for increased *Campylobacter* selection (microaerophilic and anaerobic growth); Wilkins-Chalgren blood agar (plain, non-spore forming and Gram negative supplements); Beerens agar (Beerens, 1990); Rogosa agar; MRS agar (de Man *et al.*, 1960) and Reinforced clostridial agar (all anaerobic). All blood plates contained 5% (v/v) defibrinated horse blood, with all agars, supplements and blood purchased from Oxoid, Basingstoke, UK.

Anaerobic incubation was performed in a MACS MC-1000 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) with 10% hydrogen and carbon dioxide, and 80% nitrogen atmosphere. A 2.5% CO_2 incubator (Flow Laboratories, Irvine, UK) was used for aerobic growth, and atmosphere generation jars with CampyGen and CO_2Gen sachets (Oxoid) were employed for microaerophilic and facultative anaerobic growth. Both incubators were set at 37^{o}C for 3-5 days, before colonies were picked, counted and sub-cultured for purity, prior to freezing at -80^{o}C for future microbial identification using the MIDI system (see below), or 16S rRNA gene sequencing analysis.

2.2.3 Microbial Identification System (MIDI)

All bacteria were grown on WC agar, unless they required blood (WC blood), under the same temperature and gas conditions, before injecting into their appropriate broths. The majority of patient bacterial isolates were anaerobes, while organisms that preferred aerobic or microaerophilic growth were grown on BBL Trypticase soy agar (Becton Dickinson Ltd., Oxford, UK) overnight to produce 40 mg of bacterial cells. For anaerobic growth: Gram positive organisms were inoculated into peptone-yeast basal medium with glucose (PYG) and Tween-80, while Gram negative isolates were grown in PYG without Tween-80. Fastidious organisms, such as, *C. concisus,* and other campylobacter species preferred PYG supplemented with a formate-fumarate solution. PY (no glucose) with lactate was the medium of choice for veillonellas.

FAME were extracted by saponification, methylation and a final wash step (Miller and Berger, 1989), before removal into a crimped GC vial with rubber septa (Stager

and Davis, 1992). The majority of isolates were tested using the Anaerobe Library, while aerobes were identified via the Clinical Aerobe Library. Calibrations were used before each set of samples and after each run of ten. Only calibrations that reached >99% allowed sample analysis. Blank controls were made with each batch of extractions, positive controls were also run periodically; *Escherichia coli* for aerobes and *Streptococcus stenotrophomonas* for anaerobes, while a *C. concisus* type strain was also tested regularly.

The gas chromatograph comprised a Hewlett-Packard 6890 GC Series system fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm x 25 m), a flame ionisation detector, a Hewlett-Packard model 7637A automatic sampler and a Vectra XM computer (Hewlett-Packard Ltd., Palo Alto, CA, USA). Experimental parameters were as follows: column head pressure – 60 kPa; injection volume – 2 µl; column split ratio – 100:1; septum purge – 5 ml min^{-1}; column temperature – 170-270^{o}C; injection port and detector temperatures – 300^{o}C; gases – nitrogen, ultra high purity hydrogen and zero grade laser air – "make up" gas, carrier gas and flame support, respectively.

2.2.4 DNA extraction and sequencing analysis of Campylobacter isolates

Campylobacter isolates grew well on Columbia blood agar supplemented with formate, fumarate, succinate and nitrate, which proved to be a better isolation medium for these bacteria than standard antibiotic supplemented agar (Oxoid). These organisms were also distinguishable microscopically, however, FAME profiles did not match on occasions. Consequently, all isolates suspected to be campylobacter

species due to their morphology and growth patterns, or by FAME analysis, were sent for sequencing for confirmation.

Pure campylobacter cells were swabbed into PBS and centrifuged to form a pellet, before resuspension with 450 µl molecular grade water (VWR Ltd., Leicestershire, UK) and 50 µl lysozyme (50 mg ml^{-1}) prior to incubation at 37°C for 30 min. To this suspension, 25 µl Proteinase K (Qiagen), 50 µl 20% SDS and 500 µl molecular grade water was added, together with 350 mg of 0.1 mm sterile glass beads. Two bead-beating steps (Mini-beadbeater 8, Biospec, Bartlesville, OK, USA) of 2 min, with incubations at 60°C for 10 min between these steps, facilitated release of DNA into the supernatant from the lysed cells. Cell debris was removed by centrifugation (3 min, 5000 *g*) before purification, washing and elution of the DNA in a Qiagen mini-column (DNeasy Blood and Tissue Kit, Qiagen Ltd., West Sussex, UK), according to the manufacturer's instructions.

The DNA was amplified using a campylobacter primer pair (Camp405F – GGA TGA CAC TTT TCG GAG and Camp633R – AAT TCC ATC TGC CTC TCC (Rinttilä *et al.*, 2004) with 25 µM $MgCl_2$ Mg-free thermophilic 10 x buffer, 100 µM dNTP's, and Taq DNA polymerase (1 unit). Cycling parameters were as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, 62°C for 1 min and 45 sec at 72°C, followed by a final elongation step of 10 min at 72°C. Product was run on 3% agarose gel before purification (QiaQuick PCR purification kit, Qiagen) and sequencing at the University of Dundee DNA Analysis Facility using the above forward primer (BigDye terminators in an ABI 3100 Genetic Analyser, Applied Biosystems).

2.2.5 Statistical analysis

Statistical analysis was conducted using Prism Statistical Package (Prism 4 for Macintosh, Version 4.0, Graphpad software Inc., CA, USA). All numerical data were analysed using unpaired Student's t-tests, with a normal distribution assumed (K-S test). A P-value of <0.05 was classed as highly significant, while values of <0.08 were considered to be close to significance.

2.3 Results

2.3.1 Demographics

All patients involved in the study were between the ages of 40 and 87. The mean ages of controls, GORD and BO patients were 64, 65 and 66, respectively. However, the mean age of cancer patients was 58. In this investigation, the ratio of males to females was 3:5, 3:5 and 2:6 for controls, GORD and BO, respectively. True to previously published data, the ratio of males to females in adenocarcinoma was higher (nine males, one female). The majority of these patients did not smoke or drink alcohol. Eighty-seven percent of Barrett's patients were prescribed a PPI (57% omeprazole, 43% lansoprazole), while only 50% of ADC patients were taking these drugs. Although endoscopically classed as normal, two control patients were taking PPIs, indicating reflux symptoms or gastritis. Four of the Barrett's patients had intestinal metaplasia confirmed by histology, and one had intestinal metaplasia with low-grade dysplasia. This data is summarised in Table 2.1.

Table 2.1: *Clinical details of patients taking part in study for cultural analysis of oesophageal microbiotas.*

Characteristics	Controls (8)	GORD (8)	BO (8)	ADC (10)
Sex, male:female	3:5	3:5	2:6	9:1
Age, years				
Range	46-87	55-75	48-76	40-73
Mean ± SEM	64.4 ± 4.6	65.2 ± 2.5	66.4 ± 3.3	58.4 ± 3.4
Body mass index				
19-24 (range)	2 (19.2-22.2)	4 (21.1-24.2)	1 (24.4)	6 (19.1-24.7)
25-30	2 (29.1-29.7)	3 (26.4-28.2)	4 (27.1-28.2)	2 (27.7-29.8
>30	3 (32.1-39.7)	1 (33.9)	3 (31.5-45.6)	1 (33.9)
Biopsy site, cm range				
Lower >28	8 (OGJ[a]-32)	8 (OGJ - 30)	7 (40 - 32)	9 (40 – 29)
Middle 28-24				
Upper <24			1 (24)	1 (23)
GORD inflammation				
Grade A		4		
Grade B				
Grade C		1		
Grade D		1		
Barrett's				
IM[b]			5	
Dysplasia			1	
Current treatments				
PPI	2	4	7	5
NSAIDS	1	1	3	1
Cardiac	5	5	8	4
Others	2	2	1	1
Probiotics	1	1	1	2
Smoker	0	2	1	3
Alcohol				
None	4	6	5	6
Units/week (range)	2-10	6-10	2-8	1-20
Beer	2	1	2	2
Wine	1	2	1	3
Spirits	1	1	0	1

[a] *Biopsies taken 5 cm above the OGJ.* [b] *Intestinal metaplasia.*

2.3.2 The microbial ecosystem in oesophageal disease

Bacteria were cultured on a variety of solid growth media to investigate bacterial colonisation of the oesophageal mucosa, and any alterations in microbiota composition at different stages of disease progression. With only eight samples from the control, reflux and Barrett's patients, and 10 from the adenocarcinoma patients, statistically significant variances were difficult to identify. However, as disease progressed microbial communities increased in size, although reflux patients often had reduced numbers of certain bacteria.

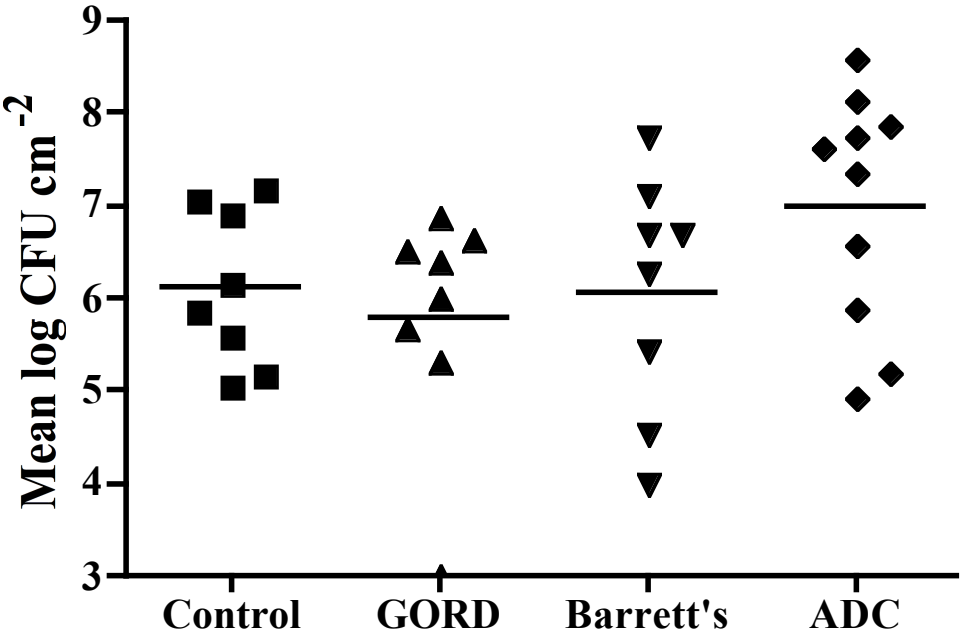


Fig. 2.1: *Total colony forming units cm^{-2} taken from WC blood agar plates grown under anaerobic conditions, data points represent the number of patients in each cohort with presence of the stated bacteria. Control (8), GORD (8), BO (8), ADC (10), bars represent the mean. Comparison of GORD with ADC gave a P value approaching significance (P = 0.06).*

Figure 2.1 shows total anaerobic CFU for all eubacteria isolated from WC blood agar plates for each biopsy sample. Although total counts were also done for aerobic and facultative anaerobes, the majority of isolates from aerobic plates also grew anaerobically.

Investigation of oesophageal microbiotas in the control group, and those with varying stages of disease, indicated that the oesophagus housed a normal microbiota established from a number of species from the oral cavity. Less common oral species such as lactobacilli and bifidobacteria, and others rarely seen in this habitat, were also isolated from the oesophagus, indicating that this organ contains its own unique community. A number of streptococci (Fig. 2.2) were present in all of the patients except for one GORD. Bacterial cell numbers ranged from $\log_{10}$ 3.4 to 7.5 CFU cm^{-2}, with a mean across all patients of $\log_{10}$ 5.5. *Streptococcus intermedius* was the main species found in this genus, being present in all controls and ADC patients, 6/8 GORD and 5/8 BO biopsies. Staphylococci (Fig. 2.3) were present in 7 control patients ($\log_{10}$ 4.1 ± 1.1 CFU cm^{-2}), however, these diminished in reflux patients ($\log_{10}$ 3.9 ± 0.2 CFU cm^{-2} (n = 4)), indicating susceptibility to acid and bile, before re-establishing in Barrett's and cancer patients ($\log_{10}$ 4.6 ± 0.9 (n = 5) and $\log_{10}$ 5.1 ± 1.5 CFU cm^{-2} (n = 6), respectively).

Actinomyces again indicated a response to refluxate in some species (Fig. 2.4, Table 2.3) with normal populations of $\log_{10}$ 4.5 ± 0.8 CFU cm^{-2} in 7 patients, with similar numbers in 4 GORD. Neisseria (Fig. 2.5) colonised the oesophagus of 5 control patients ($\log_{10}$ 3.8 ± 0.9 CFU cm^{-2}), with *N. sicca* being isolated from all subjects. Counts were reduced in GORD and BO patients, with *N. sicca* rarely being isolated. In ADC patients, these organisms were found in varying numbers, with a slight increase in mean CFU ($\log_{10}$ 4.1 ± 1.2 CFU cm^{-2} (n = 4)). Prevotella (Fig. 2.6) were present consistently throughout disease in these subjects, whereas the prevalence and absolute numbers of *Rothia denticariosa* (Fig. 2.7) decreased through each stage of disease progression - from 7 patients ($\log_{10}$ 4.5 ± 0.7 CFU cm^{-2}), to 3 ($\log_{10}$ 3.7 ± 0.3

CFU cm^{-2}). Conversely, lactobacilli (Fig. 2.8) were only identified in 3 control patients (log_{10} 3.3 ± 0.2 CFU cm^{-2}), and increased in GORD (4.4 ± 0.8 CFU cm^{-2} (n = 4)), through to Barrett's and cancer (4.6 ± 0.7 (n = 3) and 4.9 ± 1.6 CFU cm^{-2} (n = 6)) respectively. Statistical analysis of these results showed significant variation in colonisation between control and reflux patients (P = 0.07), and control and Barrett's patients (P = 0.02). Results did not significantly differ between patients with respect to bifidobacteria (Fig. 2.9), which were present in the majority of subjects. Bacteroides (Fig. 2.10) were isolated from 4 controls, 3 GORD and 4 ADC patients, with varying cell population densities, however, these bacteria were only found in one BO patient. Veillonella (Fig. 2.11) were detected in less than half of each patient group, and no significant differences were seen with respect to disease.

Fusobacteria (Fig. 2.12) occurred in low levels in each disease group, and their numbers were reduced in GORD compared to controls (log_{10} 4.5 ± 0.5 (n = 3), and log_{10} 5.4 ± 0.9 CFU cm^{-2} (n = 3), respectively). Campylobacters (Fig. 2.13) were found in only two controls, while 6 of 8 reflux patients were colonised with these bacteria (log_{10} 4.1 ± 0.8 CFU cm^{-2}). Four Barrett's and 6 ADC patients harboured campylobacters (log_{10} 5.6 ± 0.4, and log_{10} 5.3 ± 1.2 CFU cm^{-2}, respectively). Significant variation was found between GORD and BO patients (P = 0.01). Comparison of controls with BO and ADC gave P values approaching significance (P = 0.07, and P = 0.08, respectively). Both controls carried *Campylobacter concisus*, while those with GORD and ADC had *C. concisus*, *C. jejuni*, *C. coli*, *C. rectus* or a combination of two species. All Barrett's patients had *Campylobacter concisus* alone, or in combination with *Campylobacter jejuni* or *Campylobacter coli*.

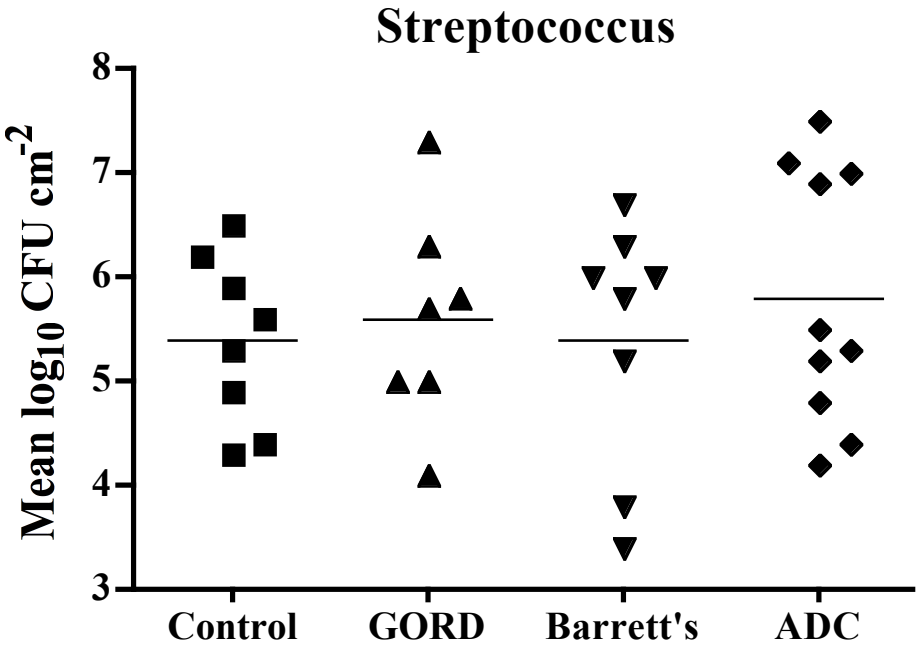


Fig. 2.2: *Log CFU cm^{-2} of biopsy showing individual counts of streptococcus for each patient. For further information refer to Fig. 2.1 legend.*

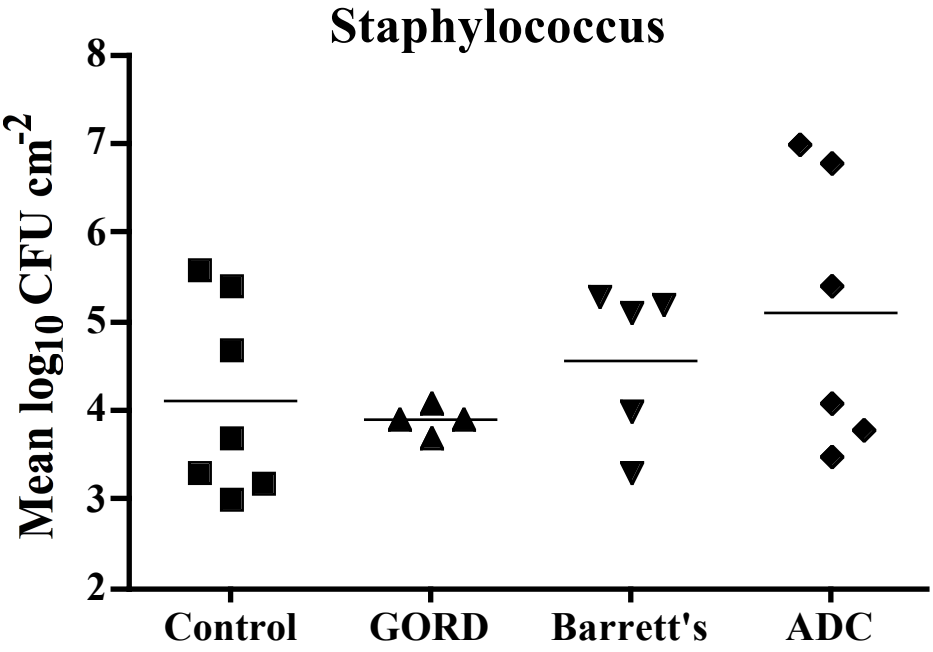


Fig. 2.3: *Log CFU cm^{-2} of biopsy showing individual counts of staphylococcus for each patient. For further information refer to Fig. 2.1 legend.*

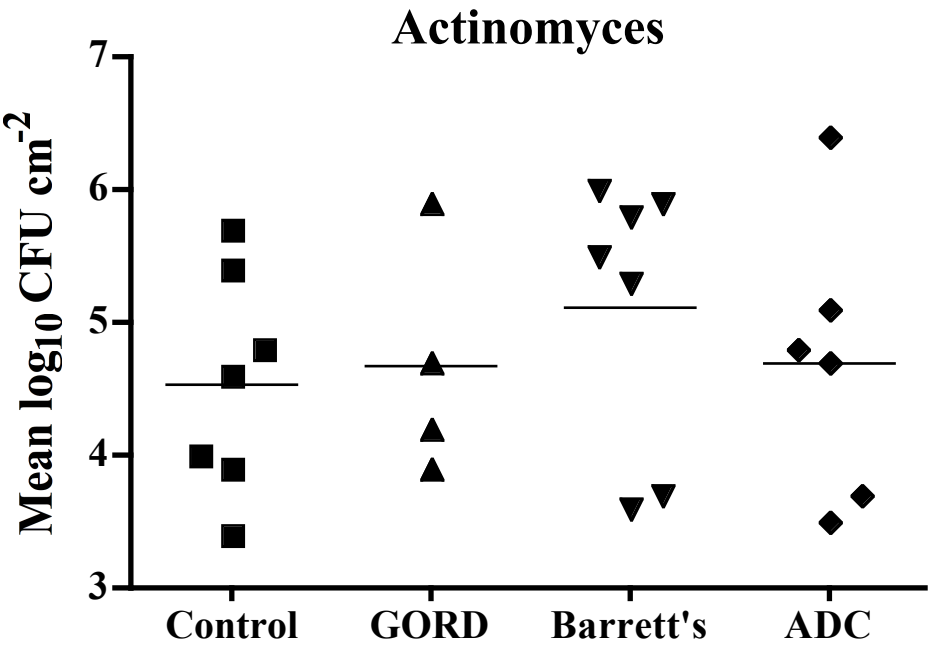


Fig. 2.4: *Log CFU cm^{-2} of biopsy showing individual counts of actinomyces for each patient. For further information refer to Fig. 2.1 legend.*

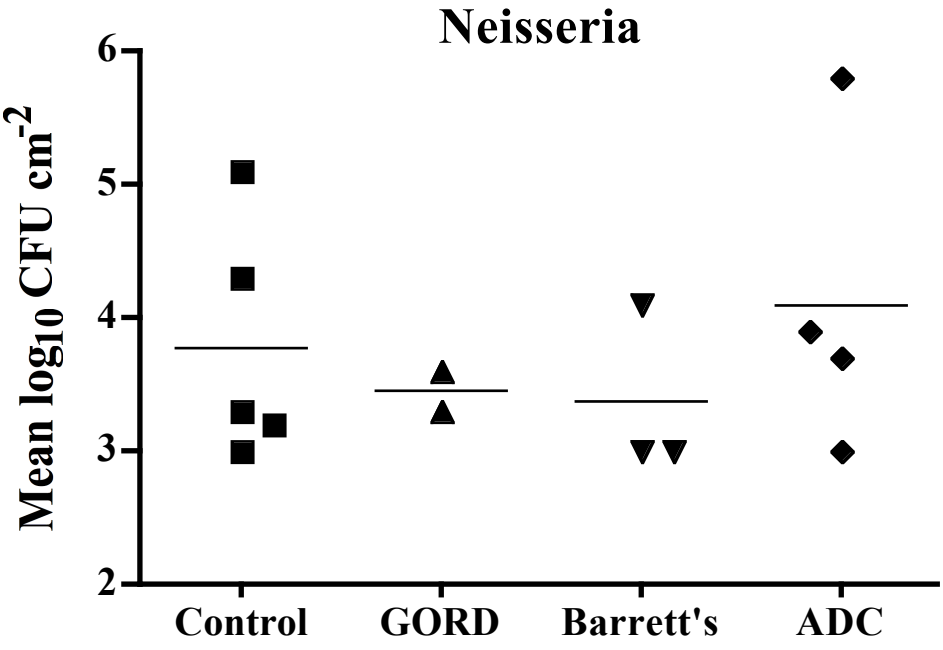


Fig. 2.5: *Log CFU cm^{-2} of biopsy showing individual counts of neisseria for each patient. For further information refer to Fig. 2.1 legend.*

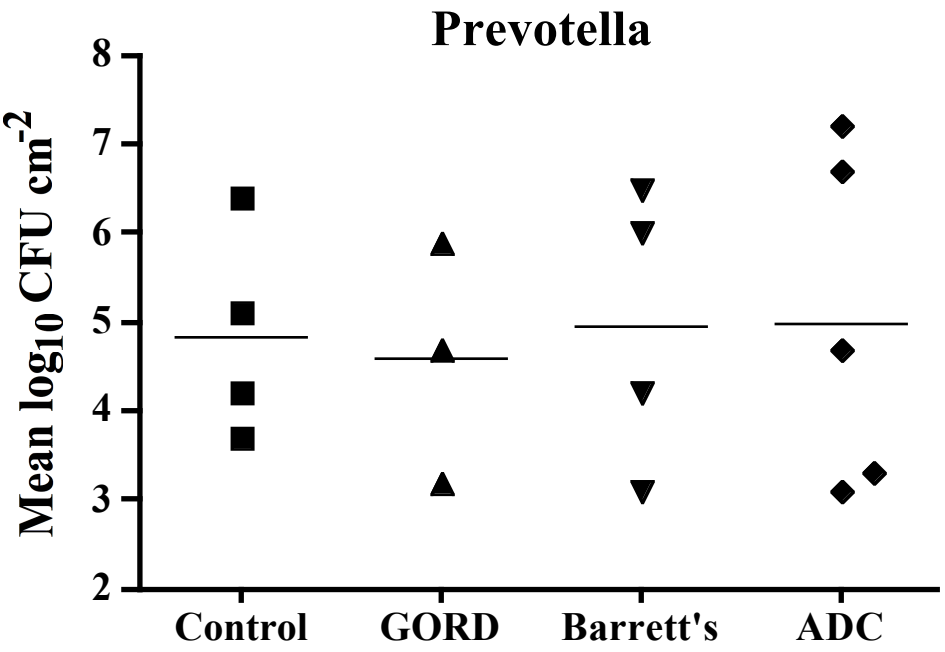


Fig. 2.6: *Log CFU cm^{-2} of biopsy showing individual counts of prevotella for each patient. For further information refer to Fig. 2.1 legend.*

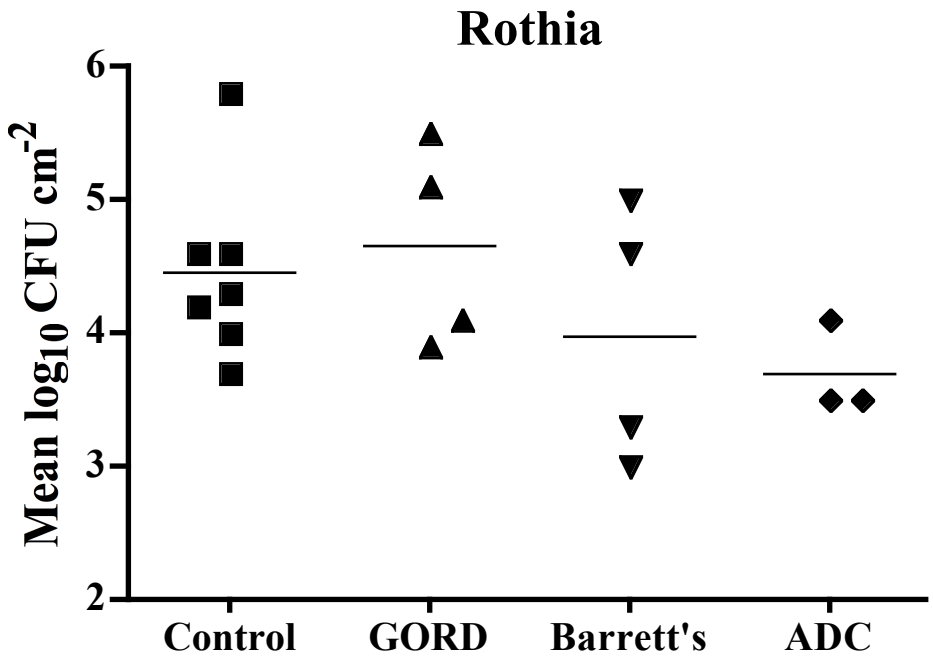


Fig. 2.7: *Log CFU cm^{-2} of biopsy showing individual counts of rothia for each patient. For further information refer to Fig. 2.1 legend.*

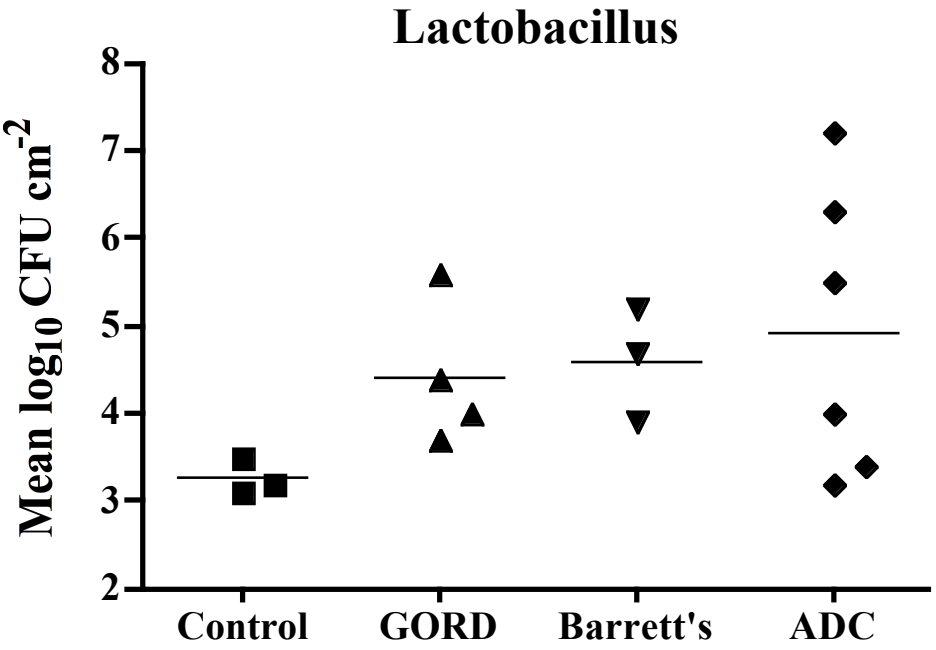


Fig. 2.8: *Log CFU cm^{-2} of biopsy showing individual counts of lactobacillus foreach patient. For further information refer to Fig. 2.1 legend. Comparison of controls with reflux and Barrett's patients gave P values of 0.07 and 0.02 respectively.*

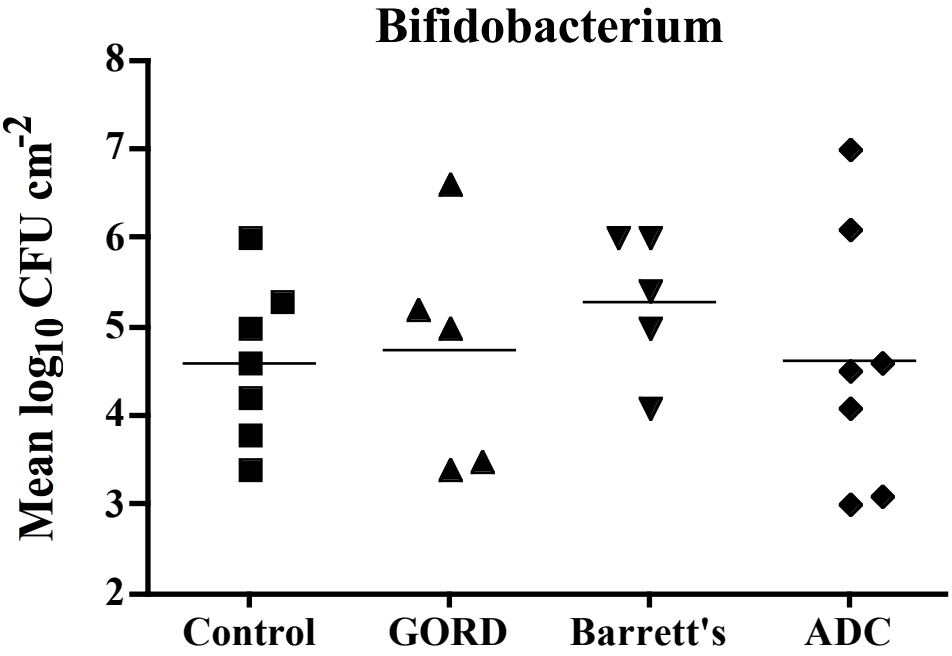


Fig. 2.9: *Log CFU cm^{-2} of biopsy showing individual counts of bifidobacteria for each patient. For further information refer to Fig. 2.1 legend.*

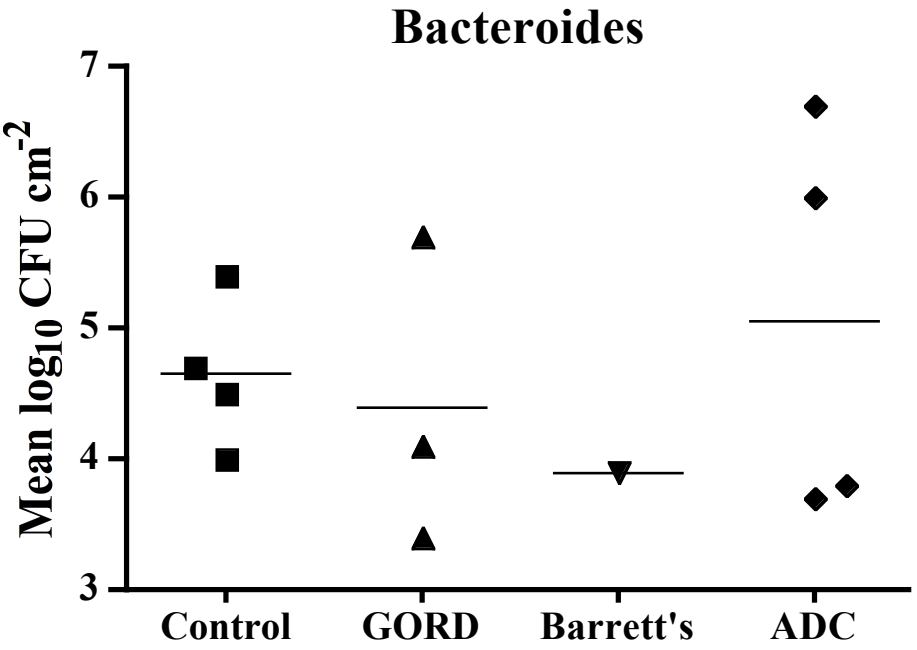


Fig. 2.10: *Log CFU cm^{-2} of biopsy showing individual counts of bacteroides for each patient. For further information refer to Fig. 2.1 legend.*

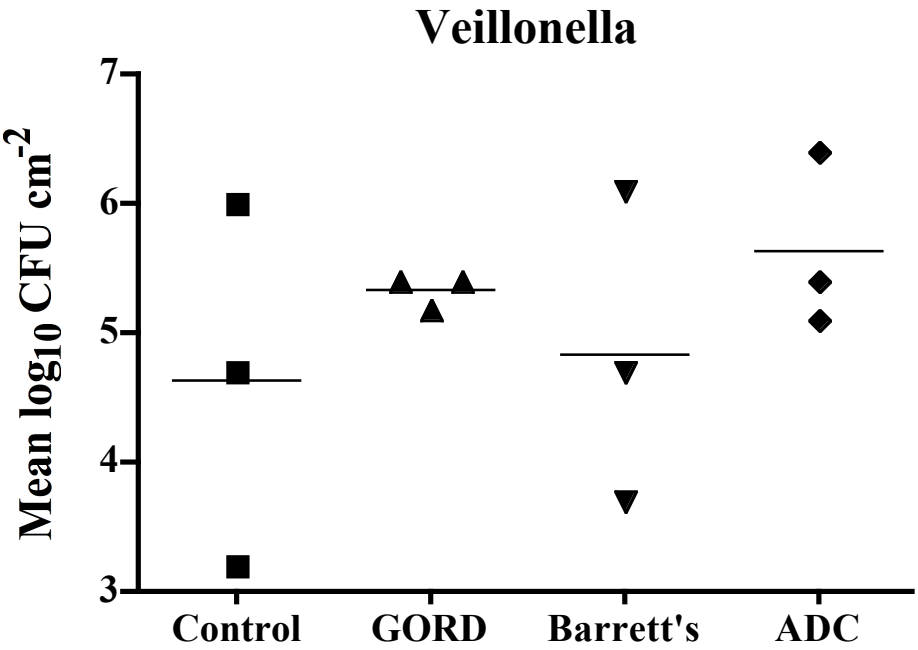


Fig. 2.11: *Log CFU cm^{-2} of biopsy showing individual counts of veillonella for each patient. For further information refer to Fig. 2.1 legend.*

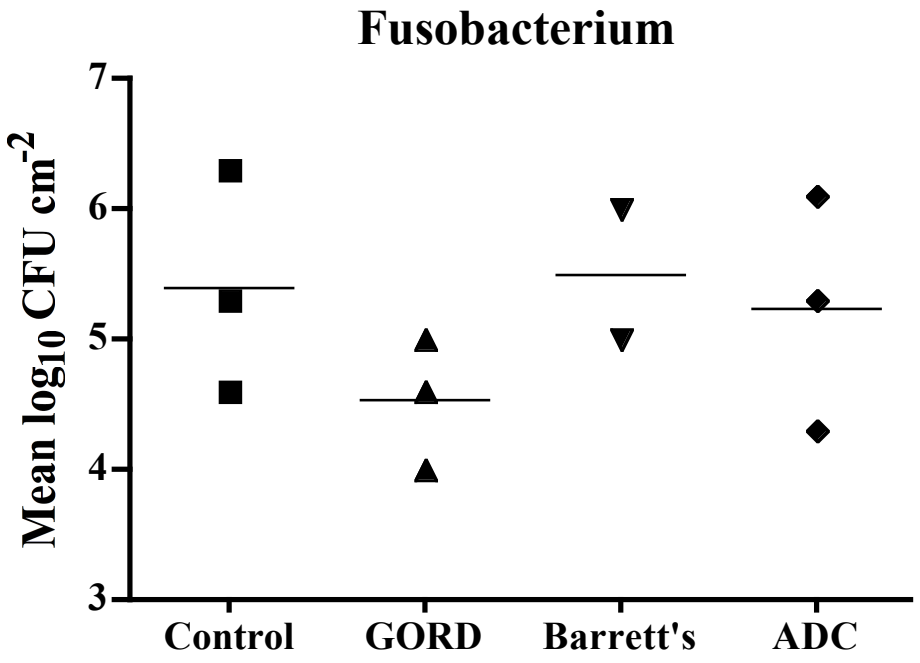


Fig. 2.12: *Log CFU cm^{-2} of biopsy showing individual counts of fusobacteria for each patient. For further information refer to Fig. 2.1 legend.*

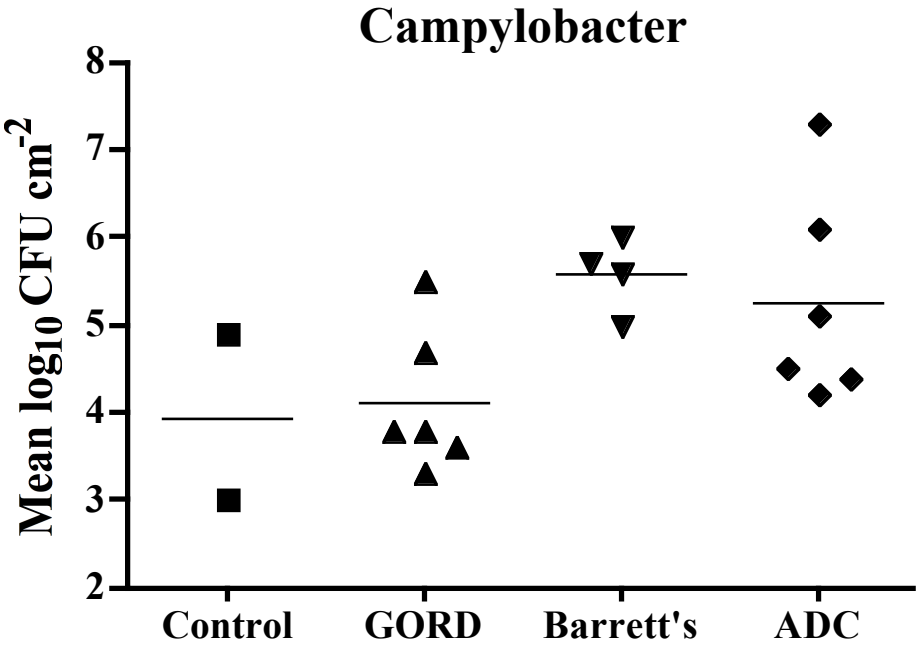


Fig. 2.13: *Log CFU cm^{-2} of biopsy showing individual counts of campylobacter for each patient. For further information refer to Fig. 2.1 legend. Changes in Barrett's patients compared with control patients reaching significance (P value = 0.07), comparison of GORD to Barrett's and ADC patients P values of 0.01 and 0.08 respectively.*

Tables 2.2 – 2.4 show all of the viable count data, with mean results for each species. In the 34 patients, 111 species representing 26 genera were isolated. In controls, 56 species belonging to 19 genera were detected, compared with 55 species and 22 genera in GORD. Sixty-one species from 23 genera were detected in BO, with 73 species and 23 genera in ADC. Although the overall microbiota composition was generally consistent in all patient cohorts, significant variations were seen at the species level in some bacterial groups, such as with lactobacilli.

***Table 2.2**: Viable counts of Gram positive cocci colonising the oesophageal mucosa [a].*

Bacteria	Normal (8)	GORD (8)	BO (8)	ADC (10)
Streptococcus				
S. mitis	5.4 ± 1.0 (7)	4.9 ± 0.5 (5)	5.2 ± 1.3 (7)	5.9 ± 1.0 (8)
S. bovis	-	-	4.1 ± 0.0 (1)	-
S. sanguinis	5.9 ± 0.0 (1)	5.9 ± 0.6 (2)	5.3 ± 1.6 (4)	4.9 ± 0.8 (2)
S. parasanguis	5.0 ± 0.7 (6)	5.4 ± 0.9 (7)	5.0 ± 1.3 (7)	6.0 ± 1.8 (4)
S. salivarius	5.1 ± 0.9 (7)	5.5 ± 1.0 (6)	5.0 ± 1.4 (8)	5.8 ± 1.1 (7)
S. intermedius	5.3 ± 0.9 (8)	5.4 ± 1.6 (6)	5.3 ± 0.8 (5)	5.6 ± 1.5 (10)
S. vestibularis	4.9 ± 1.3 (2)	4.6 ± 0.5 (2)	5.3 ± 1.0 (2)	5.1 ± 1.3 (3)
S. gordonii	4.5 ± 0.0 (1)	4.0 ± 0.0 (1)	-	4.3 ± 1.2 (3)
S. oralis	5.2 ± 0.6 (5)	4.0 ± 0.0 (1)	4.9 ± 1.1 (7)	5.4 ± 1.2 (8)
S. anginosus	4.6 ± 0.6 (2)	4.7 ± 1.9 (3)	5.8 ± 1.3 (2)	-
S. mutans	4.4 ± 0.4 (3)	-	5.7 ± 0.0 (1)	4.0 ± 0.0 (1)
S. sobrinus	5.1 ± 0.0 (1)	5.2 ± 0.0 (1)	-	3.7 ± 0.0 (1)
Staphylococcus				
Staph. capitis	-	-	4.0 ± 0.0 (1)	-
Staph. warneri	4.1 ± 1.1 (7)	3.9 ± 0.1 (4)	4.8 ± 1.0 (4)	5.2 ± 1.5 (5)
Staph. epidermidis	3.0 ± 0.0 (1)	3.4 ± 0.0 (1)	5.2 ± 0.0 (1)	5.6 ± 2.1 (2)
Staph. haemolyticus	-	-	5.0 ± 0.0 (1)	-
Enterococcus	-	-		
E. faecalis			6.7 ± 0.0 (1)	4.0 ± 0.0 (1)
Lactococcus				
L. lactis	-	-	5.3 ± 0.0 (1)	6.7 ± 1.3 (3)
Peptostreptococcus				
P. prevotii	-	-	-	4.0 ± 0.0 (1)
P. micros	-	-	-	5.5 ± 0.0 (1)
P. asaccharolyticus	-	-	-	5.2 ± 0.0 (1)
Gemella				
G. haemolysans	-	5.1 ± 0.0 (1)	5.4 ± 0.8 (2)	6.2 ± 1.2 (4)
G. morbillorum	6.7 ± 0.0 (1)	-	-	-
Micrococcus				
M. luteus	-	5.0 ± 0.0 (1)	4.0 ± 0.0 (1)	-
M. varians	-	4.7 ± 0.0 (1)	-	-
M. mucilaginosus	5.0 ± 0.0 (1)	-	-	-
M. lylae	-	-	-	5.0 ± 0.0 (1)

[a] Numbers represent the mean log_{10} CFU cm^{-2} for all patients ± SD, number in parenthesis show number of patients in each cohort harbouring this species.

***Table 2.3**: Viable counts of Gram positive rods colonising the oesophageal mucosa [a].*

Bacteria	Normal (8)	GORD (8)	BO (8)	ADC (10)
Bifidobacterium				
Bif. adolescentis	3.3 ± 0.6 (3)	-	5.2 ± 0.3 (2)	4.5 ± 1.1 (5)
Bif. angulatum	3.7 ± 0.0 (1)	5.0 ± 0.0 (1)	-	5.0 ± 2.0 (3)
Bif. infantis	-	-	-	3.0 ± 0.0 (1)
Bif. D01	-	-	-	6.1 ± 0.0 (1)
Bif. DO5	4.6 ± 0.7 (5)	4.4 ± 1.1 (4)	5.4 ± 1.3 (3)	5.6 ± 1.2 (2)
Bif. D02A	5.8 ± 0.3 (2)	-	5.0 ± 1.2 (2)	3.0 ± 0.0 (1)
Bif. breve	3.8 ± 0.0 (1)	-	5.0 ± 0.0 (1)	4.7 ± 0.0 (1)
Bif. gallinarum	4.0 ± 0.0 (1)	-	-	-
Bif. pullorum	5.3 ± 0.0 (1)	4.4 ± 0.9 (2)	-	-
Bif. bifidum	-	5.1 ± 2.5 (2)	-	-
Lactobacillus				
L. buchneri	-	4.0 ± 0.0 (1)	-	-
L. paracasei	-	-	4.5 ± 0.6 (3)	5.7 ± 2.3 (3)
L. lactis	-	-	4.0 ± 0.0 (1)	5.6 ± 0.0 (1)
L. delbrueckii	-	3.7 ± 0.0 (1)	3.0 ± 0.0 (1)	3.9 ± 0.0 (1)
L. amylophilus	-	5.8 ± 0.0 (1)	5.3 ± 0.0 (1)	-
L. casei	-	-	-	5.5 ± 0.0 (1)
L. salivarius	-	-	5.0 ± 0.0 (1)	6.6 ± 0.0 (1)
L. mali	-	4.7 ± 0.0 (1)	-	5.5 ± 0.0 (1)
L. leichmanii	-	4.8 ± 1.2 (2)	-	4.4 ± 0.0 (1)
L. plantarum	-	-	-	3.2 ± 0.3 (2)
L. agilis	-	-	-	4.0 ± 0.0(1)
L. helveticus	3.2 ± 0.0 (1)	-	-	4.5 ± 0.0 (1)
L. reuterii	-	-	-	3.5 ± 0.0 (1)
L. oris	-	-	-	5.1 ± 2.1 (2)
L. rhamnosus	-	5.5 ± 0.0 (1)	-	5.3 ± 2.7 (2)
L. brevis	-	4.7 ± 0.0 (1)	-	-
L. vaginalis	3.0 ± 0.0 (1)	-	-	-
L. gasseri	3.5 ± 0.0 (1)	-	-	-
L. animalis	2.7 ± 0.0 (1)	-	-	-
L. S01	3.2 ± 0.0 (1)	-	-	-

[a] *See footnote to Table 2.2.*

Table 2.3 cont.: *Viable counts of Gram positive rods colonising the oesophageal mucosa* [a].

Bacteria	Normal (8)	GORD (8)	BO (8)	ADC (10)
Actinomyces				
A. DO1	5.1 ± 0.0 (2)	4.8 ± 0.9 (2)	5.6 ± 0.5 (4)	4.7 ± 0.0 (1)
A. pyogenes	4.3 ± 0.7 (2)	4.1 ± 0.0 (1)	-	3.5 ± 0.0 (1)
A. odontolyticus	4.0 ± 0.0 (1)	4.9 ± 1.7 (2)	5.0 ± 1.5 (3)	5.2 ± 1.0 (3)
A. bovis	-	-	3.7 ± 0.0 (1)	-
A. israelii	-	-	3.5 ± 0.0 (1)	5.1 ± 0.0 (1)
A. naeslundii	4.3 ± 0.8 (4)	4.7 ± 0.0 (1)	5.6 ± 0.5 (2)	4.6 ± 0.3 (3)
A. slackii	-	-	-	3.7 ± 0.0 (1)
A. viscosus	5.3 ± 0.9 (2)	-	-	-
Rothia				
R. denticariosa	4.5 ± 0.7 (7)	4.7 ± 0.7 (4)	4.0 ± 0.9 (4)	3.7 ± 0.4 (3)
Clostridium				
C. glycolicum	5.4 ± 0.0 (1)	-	5.0 ± 0.0 (1)	-
C. bifermentans	-	3.3 ± 0.2 (2)	3.8 ± 0.0 (1)	-
C. malenominatum	4.7 ± 0.0 (1)	-	4.6 ± 0.0 (1)	-
C. tyrobutyricum	-	-	-	5.6 ± 0.0 (1)
C. cochlearium	-	-	-	4.0 ± 0.0 (1)
Corynebacterium				
C. matruchotii	4.0 ± 0.0 (1)	4.9 ± 1.1 (3)	7.3 ± 0.0 (1)	4.8 ± 0.3 (3)
C. naeslundii	2.7 ± 0.0 (1)	-	-	-
Propionibacterium				
P. acnes	6.3 ± 0.0 (1)	3.1 ± 0.0 (1)	5.0 ± 0.0 (1)	3.2 ± 0.1 (2)
P. granulosum	-	-	5.2 ± 0.0 (1)	-
P. propionicum	-	5.4 ± 0.0 (1)	-	3.7 ± 0.0 (1)
P. avidum	3.3 ± 0.0 (1)	-	-	-
P. D06	-	-	-	7.1 ± 0.0 (1)
Eubacterium				
E. combesii	-	-	5.0 ± 0.0 (1)	-
E. limosum	-	5.0 ± 0.0 (1)	4.8 ± 0.0 (1)	5.5 ± 0.0 (1)
E. brachy	-	-	-	4.0 ± 0.0 (1)
E. D27	-	6.0 ± 0.0 (1)	-	-

[a] *See footnote to Table 2.2.*

***Table 2.4**: Viable counts of Gram negative bacteria colonising the oesophageal mucosa [a].*

Bacteria	Normal (8)	GORD (8)	BO (8)	ADC (10)
Bacteroides				
B. coagulans	5.4 ± 0.0 (1)	3.8 ± 0.5 (2)	3.9 ± 0.0 (1)	4.7 ± 1.7 (3)
B. vulgatus	4.5 ± 0.0 (1)	-	3.9 ± 0.0 (1)	-
B. D53	-	5.7 ± 0.0 (1)	-	-
B. D25	4.4 ± 0.5 (2)	-	-	6.0 ± 0.0 (1)
Prevotella				
P. tannerae	4.8 ± 1.2 (4)	5.9 ± 0.0 (1)	4.9 ± 1.6 (2)	5.0 ± 2.0 (5)
P. nigrescens	-	3.3 ± 0.0 (1)	6.5 ± 0.0 (1)	-
P. melaninogenica	-	4.7 ± 0.0 (1)	3.0 ± 0.0 (1)	-
P. loescheii	-	-	4.4 ± 0.0 (1)	-
P. buccalis	-	-	-	5.0 ± 0.0 (1)
Campylobacter				
C. concisus	4.0 ± 1.3 (2)	4.5 ± 1.2 (5)	5.7 ± 0.5 (4)	4.5 ± 0.4 (2)
C. jejuni	-	4.8 ± 0.0 (1)	5.1 ± 1.2 (2)	5.8 ± 0.5 (2)
C. coli	-	3.9 ± 0.5 (3)	4.0 ± 0.0 (1)	7.3 ± 0.0 (1)
C. rectus	-	-	-	4.4 ± 0.0 (1)
Fusobacterium				
F. alocis	4.5 ± 0.0 (1)	4.6 ± 0.0 (1)	-	-
F. russii	5.8 ± 0.7 (2)	4.0 ± 0.0 (1)	5.7 ± 0.5 (2)	5.0 ± 0.0 (1)
F. nucleatum ss. vincentii	-	-	3.5 ± 0.0 (1)	6.0 ± 0.0 (1)
F. nucleatum	-	5.0 ± 0.0 (1)	-	5.5 ± 0.0 (1)
F. necrophorum	-	-	-	4.3 ± 0.0 (1)
Faecalibacterium				
F. prausnitzii	-	-	5.3 ± 0.2 (3)	-
Escherichia coli	3.7 ± 0.0 (1)	4.6 ± 0.0 (1)	-	-
Neisseria				
N. mucosae	4.1 ± 1.2 (2)	3.3 ± 0.0 (1)	3.0 ± 0.0 (1)	3.5 ± 0.6 (2)
N. flavescens	4.0 ± 0.0 (1)	3.6 ± 0.0 (1)	3.0 ± 0.0 (1)	-
N. sicca	3.9 ± 1.0 (5)	-	4.0 ± 0.0 (1)	3.7 ± 0.0 (1)
N. subflava	-	-	3.0 ± 0.0 (1)	-
N. elongata	4.0 ± 0.0 (1)	-	-	5.8 ± 0.0 (1)
N. cinerea	4.0 ± 0.0 (1)	-	-	-
Veillonella				
V. atypica	6.0 ± 0.0 (1)	-	4.6 ± 0.0 (1)	5.9 ± 0.7 (2)
V. parvula	4.0 ± 1.0 (2)	5.3 ± 0.1 (3)	4.9 ± 1.7 (2)	5.1 ± 0.0 (1)
Gardnerella				
G. vaginalis	6.1 ± 0.7 (2)	4.6 ± 0.7 (3)	5.0 ± 1.2 (4)	4.7 ± 0.0 (1)
Leptotrichia				
L. buccalis	5.0 ± 0.0 (1)	5.0 ± 0.0 (1)	3.7 ± 0.5 (2)	4.8 ± 0.3 (2)
Arcobacter				
A. cryaerophilus	-	5.1 ± 1.3 (2)	-	-

[a] *See footnote to Table 2.2.*

2.3.3 Bacterial colonisation of the oesophagus

Figures 2.14 – 2.16 show the percentage that each bacterial group constitutes in each patient cohort (control, GORD, BO, ADC). The data provide a global view of the microbial composition of each disease group. Figure 2.14 identifies all groups in each microbiota, however, those genera constituting 6% or less each of the control patients were classified as Group B, while those composing less than 2% of the control microbiota were placed in Group C. This information is presented in Figs. 2.15 and 2.16, respectively.

Figure 2.14 identifies the major organisms isolated, constituting between 6% and 13% of the microbiota in the control subjects. Streptococci were similar in controls and diseased patients, while bifidobacterial numbers went down from 10% of the control population to 7% in each disease group. Rothia constituted 9.5% of the control microbiota, reducing to 6% in GORD, 4.5% in BO, and 2.5% in ADC. Neisseria made up 6% of the control population, falling to 2-3% in the disease cohorts. Group B organisms constituted 25-29% of the microbiota, while Group C accounted for ca. 10% of the total normal microbiota, increasing to 27% in GORD, 25% in BO and 30% in ADC.

In group B (Fig. 2.15), bacteroides comprised 6% of the microbiota in controls, 1% in BO and 4.5% in ADC. Gardnerellas were found in 50% of BO patients, constituting 6% of the total microbiota. This proportion was reduced to 1% in ADC, where the organisms were found in only 1 patient. As shown in Fig. 2.8, lactobacilli increased with disease progression, comprising 3% of the control microbiota, and 7% in ADC.

Group C bacteria (Fig. 2.16) varied through the disease cohorts, with 6 genera isolated in controls, and 8 in GORD, due to the appearance of *Arcobacter* and *Eubacterium* species. Barrett's patients had 9 genera in Group C, with *Faecalibacterium prausnitzii* being present in 3 of the 8 patients, but not in any other patient group. ADC also had 9 different genera, with peptostreptococci colonising 3 of the 10 subjects in this cohort. Campylobacter colonisation increased from 2% of the control microbiota to 8% in GORD, 6% in BO, and 7% in ADC. This genus showed the largest percentage increase of all bacterial species in these patients. Gemella increased from 2% in controls to 5.5% in ADC, while corynebacteria increased slightly in GORD patients compared to the other two disease cohorts (2% to 5%). *Escherichia coli* was found only in 1 control and 1 GORD patient (1% each), while eubacteria were found in GORD, BO and ADC patients, but not in any of the controls. *Arcobacter cryaerophilus*, a member of the *Campylobactericeae* family was only found in two reflux patients, constituting 3.3% of the total GORD microbiota. *Lactococcus lactis* was found in 1 Barrett's patient, however, this species comprised 5% of the ADC microbiota, being isolated from three of these patients, with a high mean CFU ($\log_{10}$ 6.7 cm^{-2}). Enterococci were similarly only found in BO and ADC patients.

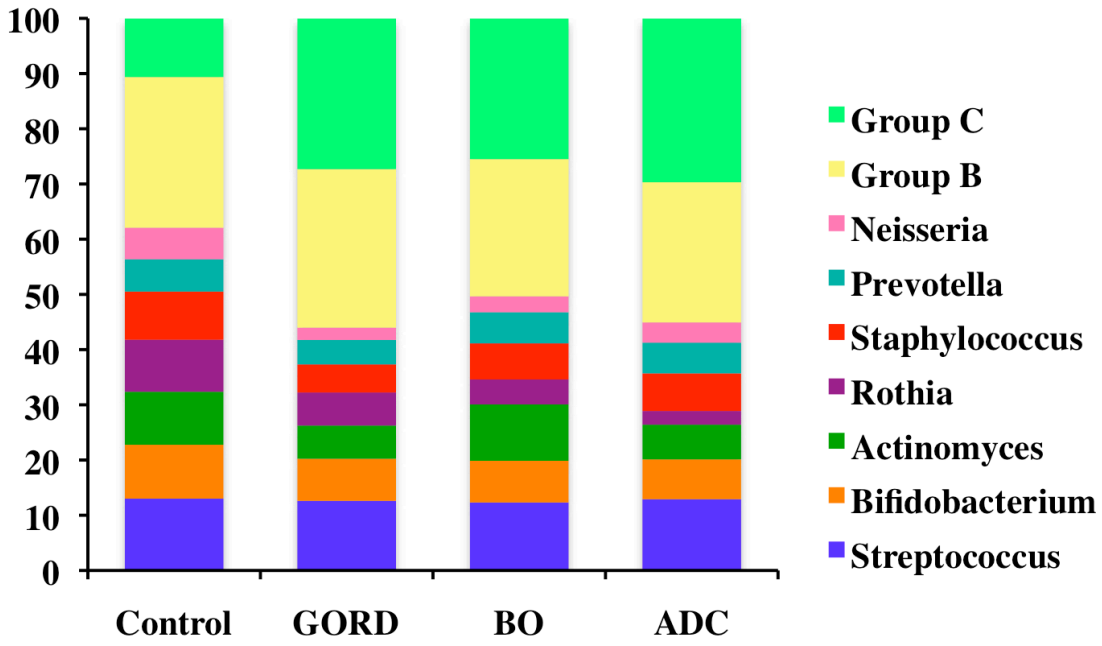


Fig. 2.14: *Global percentages of the total bacteria found throughout the patient groups (8 control, 8 GORD, 8 BO and 10 ADC). Bacteria in Groups B and C are represented in Figs. 2.15 and 2.16.*

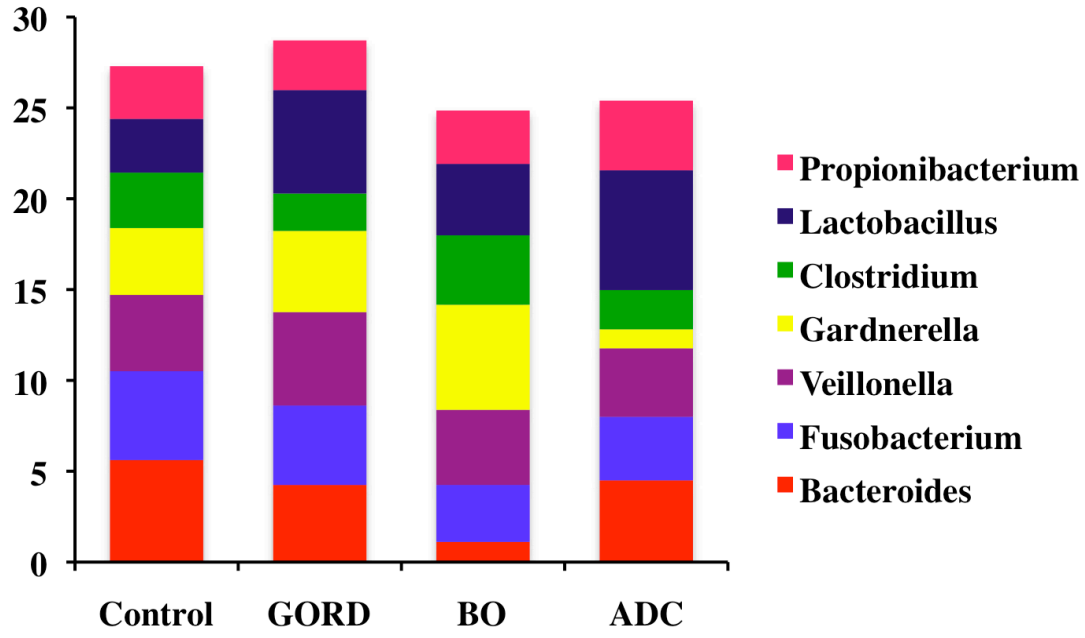
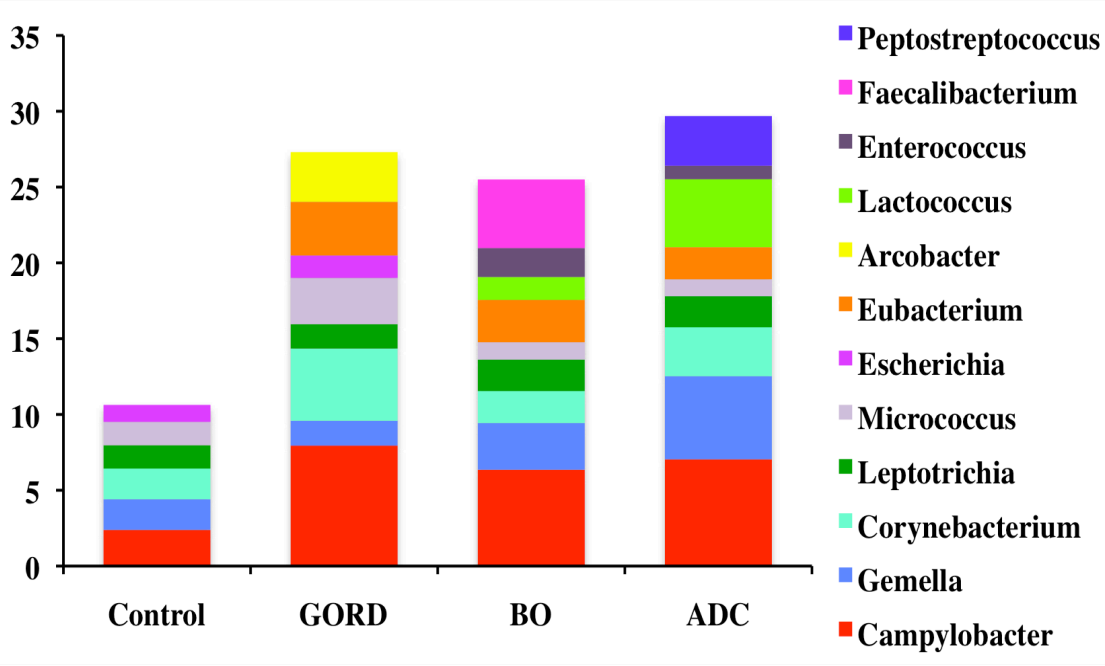


Fig. 2.15: *Percentage separation of organisms in Group B. These genera each constituted 6% or less of the total control population.*



2.16: *Percentage separation of organisms in Group C. These genera constituted less than 2% each of the total control population.*

2.4 Discussion

The research in this chapter aimed to expand on the published data investigating the microbiota colonising the oesophageal mucosa. Previous research looked at healthy patients compared with those diagnosed with GORD and BO. This research used culturing techniques to look more closely at these patients, and additionally recruited subjects diagnosed with adenocarcinoma of the oesophagus.

This study represents the first investigation of the oesophageal microbiota in ADC patients. Ten subjects with oesophageal ADC were analysed in this first part of the study, with 34 ADC and squamous cell carcinoma subjects being investigated throughout the project, both through culturing and molecular techniques.

Bacterial populations play an important role in the maintenance of local gut physiology, and often disease pathology, as indicated by the development of

inflammatory bowel diseases (Macfarlane *et al.*, 2009). It is hypothesised that the oesophageal microbiota is chiefly acquired from that of the oral cavity, and in disease, may also contain microorganisms originating from the stomach due to regurgitation of its contents during reflux. Oesophageal physiology, and alterations in cell morphology acquired during reflux disease, may have an effect on the host microbiota, leading to ADC development.

Results from this part of the study indicated a shift in the oesophageal microbiota during disease progression. Five bacterial phyla were identified in all 34 patients: Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes and Fusobacteria. In these phyla, 11 genera of Firmicutes were found: *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Gemella*, *Lactobacillus*, *Veillonella*, *Enterococcus*, *Clostridium Lactococcus, Faecalibacterium* and *Eubacterium.* Seven genera of Actinobacteria were identified: *Bifidobacterium*, *Actinomyces*, *Rothia*, *Gardnerella, Micrococcus*, *Propionibacterium* and *Corynebacterium*. The Bacteroidetes, prevotella and bacteroides were found, while the Proteobacteria *Campylobacter*, *Arcobacter* and *Neisseria* were identified, along with *Fusobacterium* and *Leptotrichia* from the phylum Fusobacteria. These results concur with previous studies investigating the oesophageal microbiota in health and disease (Pei *et al.*, 2004, 2005; Macfarlane *et al.*, 2007; Yang *et al.*, 2009). In total, 111 species representing 26 genera were isolated, however, in controls only 19 genera and 56 species were detected, compared with 23 genera and 73 species in all ADC patients.

The data for controls is similar to that of the 4 patients in the Pei *et al.* (2004) study of the healthy oesophagus. Research into the oesophageal microbiota performed by Pei *et al.* (2004, 2005) and Yang *et al.* (2009) used PCR clone libraries and statistical

clustering analysis methods. There are arguments against using traditional culture, with over 50% of the 700 species in the oral cavity being uncultivable, or more correctly, difficult to culture (Aas *et al.*, 2005). However, sequenced DNA could have multiple origins: viable bacteria adhering to the mucosal surface; transient organisms moving through the oesophagus, whether live or dead; and DNA fragments from dead bacterial cells. Nevertheless, results from these molecular based studies were similar to those obtained by Macfarlane *et al.* (2007) and the present study, with comparable pattern shifts in the microbiota being observed during disease progression. Consequently, the advantages of molecular techniques compared to culturing may not be as significant as previously thought.

The phylum and genus TM7 was isolated from patients in the molecular-based studies (Pei *et al.,* 2004, 2005; Yang *et al.* 2009). This genus has previously been recovered from a variety of habitats, ranging from hydrothermal sediments to the human mouth (Luo *et al.*, 2009). A study by Marcy *et al.* (2007) used single cell genetic analysis techniques and FISH to identify and sequence TM7, before comparative analysis to identify possible similarities with other genera. This has given some insight to the biochemistry, and possible virulence of this genus. They identified this bacterium from an oral biofilm, discovering a possible Gram positive phenotype with thick rod morphology. This study found a small number of genes with similarities (>60%) to fusobacteria*,* clostridia and bacilli, however, these may have originated from DNA contamination. Additionally, genes encoding type IV pilus proteins were identified, suggesting gliding motility (Marcy *et al.*, 2007). TM7 species may be an important component of the oesophageal biofilm, with a significant role in disease pathogenesis; however, much is still to be discovered about this bacterium. With no TM7 cultured to date, it is not known whether the

TM7 sequences found during studies of the oesophageal microbiota (Pei *et al.*, 2004, 2005; Yang *et al.*, 2009) are from viable colonising organisms.

Results obtained in studies described in this chapter did not reveal any specific organisms that were present in the majority of disease phenotypes that did not occur in any of the healthy controls. Thus, a single aetiologic agent could not be linked to oesophageal disease. Nevertheless, differences in the prevalence of particular bacterial groups and relative bacterial numbers were observed, with the microbiota becoming increasingly Gram negative. This was particularly evident with staphylococci, actinomyces and neisseria, which were reduced in GORD. However, as oesophageal cell morphologies continued to change with disease progression, becoming columnar, and then metaplastic/dysplastic, the prevalence of these species reverted to that found in normal patients. This data indicates that changes in the extracellular environment, cell surface structure and gastric secretions could affect these organisms, rendering them less able to colonise inflamed mucosa. These organisms may also be particularly acid and bile acid-sensitive. However, with the addition of other acid-resistant species forming microcolonies and biofilms on the modified host cells, they may be able to repopulate the mucosa.

As disease progressed, lactobacillus diversity increased, with 13 different species being detected in ADC alone. Three control patients harboured lactobacilli at levels of $\log_{10}$ 3.0 CFU cm^{-2}, while 4 GORD, 3 BO and 6/10 ADC patients had numbers as great as 7.5 $\log_{10}$ CFU cm^{-2}. Lactobacilli are present in a large number of fermented foods; therefore, many of these organisms could simply have been in transit through the upper GI tract. *Rothia denticariosa* displayed a reduced ability to colonise the oesophageal mucosa during disease, with a proportional reduction both in the

number of patients colonised and CFU cm^{-2} of tissue. This is converse to the results of Yang *et al.* (2009), where significantly higher numbers of this organism were found in disease patients.

The recent study by Yang *et al.* (2009) used statistical clustering analysis to further investigate their results. Two distinct microbial populations were detected, distinguishing healthy individuals from those with oesophageal disease. This group designated these 'Type I' and 'Type II' microbiomes, with the former being found in healthy patients (92%), and the latter in those with GORD (58%) and BO (60%). The type I microbiota was characterised by having a majority of streptococcal species; 78% compared with 29% in the type II microbiota. The percentage of gemellas was reduced in the abnormal oesophagus of type II microbiome patients, while Gram negative species were increased. The genera *Prevotella*/*Bacteroides*, *Campylobacter*, *Neisseria* and *Fusobacterium* increased significantly in the abnormal microbiota, with total Gram negative species increasing from 14.9% to 53.5% between type I and type II. Finally, 16.3% of the type I microbiota was comprised of anaerobic/microaerophilic organisms, compared with 61% of the type II microbiome (Yang *et al.*, 2009). This study is in line with our hypothesis, indicating that the normal (type I) microbiota is predominantly established from the oral cavity, while in diseased patients, the biofilm becomes increasingly colonised with gastric bacteria and Gram negative species, signifying that it is not a single genus or species that stimulates disease progression, but a whole shift in the microbiome.

The global population analyses done in this study found a shift in microbiota composition between control and abnormal patients. Figures 2.14 – 2.16 show the

key changes in percentage populations of each genus with Fig. 2.14 identifying the 7 most abundant genera which comprised 6-13% of the control population. In contrast to the study by Yang *et al.* (2009), streptococci constituted similar proportions of the microbiome in all patients, with an opposing shift in proportions of bifidobacteria, rothia, bacteroides and gemella. Similar to the study by Yang *et al.* (2009), lactobacilli, campylobacter, leptotrichia and peptostreptococcus populations increased with disease progression, suggestive of this type II microbiome.

Although these two studies do not show a complete correlation of results, the proposal of a whole shift in biofilm composition, rather than one specific pathogen being responsible for disease progression, is plausible. The results of all of these studies into the oesophageal microbiota do not identify one organism present only in disease; however, campylobacters manifested the greatest increase during disease progression in both studies using culture techniques (Macfarlane *et al.*, 2007; this investigation). The increased presence of pathogenic nitrate-reducing *Campylobacter* species in disease patients is of some concern. These bacteria are fastidious, slow growing microaerophilic organisms that often require special filtration methods for their isolation. Additionally, they are sensitive to many of the antibiotics used in selective culture media for the isolation of *C. jejuni* and *C. coli*. Therefore, a new medium was designed to selectively grow these organisms, in which no antibiotics were added and the preferred energy sources formate, fumarate, nitrate and succinate were provided to enhance the growth of *C. concisus*, the main species found in BO patients by Macfarlane *et al.* (2007). The use of this medium proved successful, in that it was able to isolate these organisms with greater ease under microaerophilic and anaerobic conditions. *Campylobacter concisus* was the main campylobacter found in this study, however, some patients that harboured this species were also

colonised with one or more of *C. jejuni*, *C. coli* or *C. rectus*. *Campylobacter concisus* has previously been associated with periodontal diseases in humans (Tanner *et al.*, 1979), and has been linked to enteritis in children (Lindblom *et al.*, 1995). The organism was found in multiple areas of the healthy mouth (Aas *et al.*, 2005), indicating that it may be a potential pathogen found in the normal commensal microbiota of some humans.

As discussed in Section 1.5.5, nitrate reduction by oral bacteria such as veillonella, rothia and actinomyces, may provide a mechanism for further oesophageal damage, through the production of nitric oxide during gastric reflux. The increased prevalence of nitrate reducing *C. concisus*, not only in BO patients as found previously (Macfarlane *et al.*, 2007), but also in those with GORD and ADC, may increase the mutagenic effects of refluxate alone at the OGJ. *Campylobacter concisus* has a varied virulence potential, and some strains have been shown to invade Hep-2 (human epidermal) cells faster than *C. jejuni* (Russell and Ward, 1998), producing cytotoxic effects similar to those of *H. pylori*. In the pathology of gastric adenocarcinoma, *H. pylori* has been identified as the main aetiologic agent, however, this is dependent on the strain's virulence status, in particular, possession of the CagA pathogenicity island (Lochhead and El-Omar, 2008). In the present study, although campylobacters were increasingly isolated in disease, they were also isolated from two healthy controls; therefore, virulence genes may be a key factor in disease aetiology. Further research is essential to fully sequence these isolated campylobacters, and investigate the presence of pathogenicity islands and consequently, virulence potential.

The oral pathogens *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis* can co-adhere *in vivo*, forming what is known as the "Red Complex" (Socransky *et al.*, 1998). The growth of these organisms in close juxtaposition on mucosal surfaces amplifies their pathogenicity to levels greater than possible individually. The polymicrobial disease theory suggests that this complex is accompanied by a variety of organisms (Section 2.1.3) often not found in healthy individuals. Therefore, the presence of these virulent organisms may modify community behaviour to enhance bacterial survival and virulence.

Oral microorganisms bind to saliva adsorbed onto surfaces in the oral cavity; the varying surfaces in the mouth present an array of receptors (immunoglobulin, α-amylase, fibronectin, lactoferrin). These proteins preferentially bind certain species compared with others, accounting for the variations in biofilm communities in different areas of the mouth (Socransky *et al.*, 1998). As tissue becomes diseased these surface receptors can become altered. This provides an increased proteinaceous environment supporting the growth of proteolytic organisms, and thus escalating the disease pathway due to further alterations from a commensal, healthy community. Hence, in the case of oesophageal disease the presence of campylobacters alone may not initiate the disease process but alterations in oesophageal cell morphology, and consequently the adhering microbiota, might lead to the development of a pathogenic community.

Interestingly, a recent mouse model study (Strachan *et al.*, 2008) infected 5 male and 4 female mice with *C. jejuni* under maintained environmental conditions, investigating a possible sexual dimorphism in this genus. Results found statistically increased colonisation of male mice compared with females by this species,

indicating that physiological differences associated with gender affect campylobacter colonisation. Men are at increased risk of developing oesophageal ADC; therefore, this data gives credence to the hypothesis of campylobacter being an aetiologic agent in oesophageal disease progression, possibly in synergy with other species.

This initial study of the oesophageal microbiota in healthy, GORD, BO and ADC patients has not identified a specific aetiologic agent. However, it has given credence to the hypothesis of bacterial involvement in oesophageal ADC development. Due to the relatively small number of individuals in each patient cohort, a common pattern could not be identified and environmental factors such as PPI use could not be assessed. The patients recruited for this study had a diverse age range compared with the molecular-based studies by Pei *et al.* (2004, 2005) and Yang *et al.* (2009) which recruited from an all male veteran centre with the majority of volunteers over the age of 70 years. The patient demographics in these three studies may have affected their results, due to the general shift in the commensal microbiota with age (Hopkins *et al.*, 2001).

The proposal that Barrett's oesophagus and adenocarcinoma may have a polymicrobial disease aetiology is plausible, with many human diseases being due to multiple species. *Campylobacter concisus* chiefly colonised patients in juxtaposition with other campylobacter species, leptotrichia, arcobacter and fusobacteria. These organisms are all known pathogens, and therefore, when complexed together in a biofilm could have amplified virulence effects, similar to that of the oral "Red Complex".

The human microbiota is a highly complex system, although there is a baseline of common bacteria, microbial composition is most likely completely unique to each individual. Presence of a type I or type II microbiota (Yang *et al.*, 2009) could be due to the effect of refluxate, or individuals may habitually contain one or the other microbiome phenotype. Data from all of these studies on the oesophageal community do however, indicate a shift in composition with disease progression, in that it becomes increasingly diverse and Gram negative based. There are many environmental factors that could initiate this modification, including other disease status, antibiotic use, and life-style choices such as diet. Further research into the microbiota is required, with a larger cohort of patients in each disease group. Moreover, experimentation with individual bacteria, such as campylobacter, is essential to understand both their virulence potential, and consequently, their interactions with the host.

Chapter 3

Molecular analysis of oesophageal biofilm communities

3.1 Introduction

The previous chapter reported on the bacteriological analysis of four patient cohorts (controls, GORD, BO, ADC), and identified the predominant viable organisms colonising the oesophageal mucosa in health and disease. These results, used together with earlier data obtained by Macfarlane *et al.* (2007) were used to design assays for real-time PCR. Molecular techniques such as this allow large numbers of patient samples to be quantitatively analysed at a greater rate than is possible with culture-based microbiology. The use of molecular technologies is expanding greatly, and will continue to do so in the future. It is inconceivable, at this time, to know what will be learned about human and environmental microbial ecosystems. The study of human genetics has expanded greatly in recent years, with the establishment of the Human Genome Project, and this has yielded important insights into human diseases, and consequently, potential treatments. With the Human Microbiome Project (National Institutes of Health) underway, the relationship of the host with the GI ecosystem will be better understood, allowing new areas of research to be developed.

Experiments with mono- and dizygotic twins, compared with unrelated individuals, show that host genotype has a considerable impact on the gut community (Zoetendal *et al.*, 2001; Stewart *et al.*, 2005). The interplay between host and microbial genetics seems to involve cross-talk (Bry *et al.*, 1996; Hooper and Gordon, 2001; Hooper *et al.*, 2002), allowing management of nutrient production, metabolism and immune regulation. The latter has been understood for many years, however, researchers are currently finding the genetic basis for the former.

Insight into these intricate interactions is of great relevance to the aetiology of gut disorders, especially gastrointestinal cancers. The involvement of *H. pylori* in gastric ulcers leading to ADC is one of the most significant findings in this area, with much research dedicated to these interactions between host and bacteria. As discussed in Section 1.2.8, variation in the immune response to infection is dependent on host genetics. This study aims to further identify the mucosal biofilms present in control, GORD, BO and ADC patients using real-time PCR. This technology will also be employed to assess cytokine responses to the oesophageal microbiota, aiming to identify links between mucosal populations, the inflammatory process and the potential progression to, or maintenance of, adenocarcinoma.

3.1.1 Real-time PCR

The traditional polymerase chain reaction (PCR) technique was devised nearly 40 years ago (Kleppe *et al.*, 1971), however, the discovery of heat stable taq polymerase, isolated from the bacterium *Thermus aquaticus*, led to the full development of this technique during the following decades (Mullis, 1990). In 1993, Dr Kary Mullis was awarded the Nobel Prize in Chemistry for the invention of PCR. This technique allows logarithmic amplification of short DNA sequences, using primers (short sequences of ca. 20 base pairs) which bind to their complementary bases, extending upon heated reaction to produce multiple copies of double-stranded DNA. Continuing from this revolutionary technology, Higuchi and co-workers at Roche Molecular Systems constructed a system that allows the real-time analysis of this amplification reaction (Higuchi *et al.*, 1993). In contrast to traditional PCR, where product is analysed at end-point using agarose gel electrophoresis, during the real-time PCR reaction, a video camera measures the level of

bound fluorescent dye during each cycle, facilitating quantification of double-stranded DNA relating to the exact starting quantity. Originally, ethidium bromide allowed visualisation of fresh double-stranded DNA, however, new and safer probes such as TaqMan and Sybr green are now available. There are two techniques available for quantification in real-time PCR: absolute and relative. The former relies on a serially diluted standard curve with specific input values for gene copy number, while the latter determines only the fold-difference in material.

In this study, standard curves were prepared for each target gene employing cloning techniques to produce a plasmid with a known value, as illustrated in Fig. 3.1. This shows an example of a real-time PCR amplification report, showing graphs of relative fluorescence units (RFU) per cycle, and the associated standard curve plotted from these results. These graphs were taken from a *H. pylori* assay in this study, with a serially diluted plasmid of 10^6-10^1 molecules of target μl^{-1}.

Real-time PCR offers a number of advantages over traditional PCR, such as collection of data during the exponential growth phase, which minimises the use of post-PCR processing (gel electrophoresis). The increase in relative fluorescence units is directly proportional to the number of amplicons generated, giving quantitative rather than qualitative data for presence of the gene, and therefore, bacterial load in the starting material. Real-time PCR is a sensitive technique, although it is not necessarily more specific or sensitive than traditional culture. However, in a study such as this, where such large sample numbers are required, real-time PCR provides a time saving and reliable technique for analysis of mucosal populations.

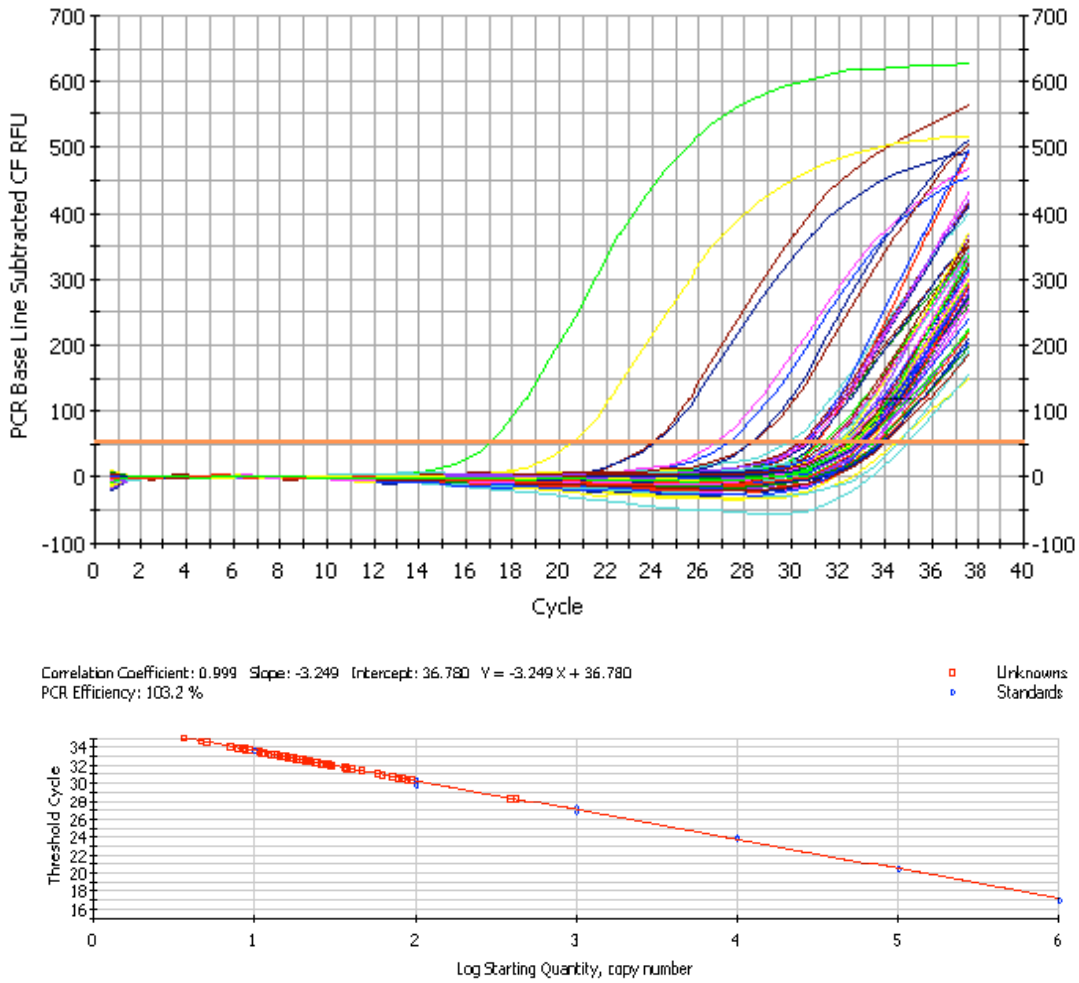


Fig. 3.1: *Real-time PCR amplification graph (top) and associated standard curve (bottom)*. The *amplification graph shows the exponential phases for the serially diluted plasmid, with the standard curve plotted from the baseline, identifying the gene copy number for each biopsy sample (red squares). This image is taken from the H. pylori assay in this study.*

3.1.2 Issues with campylobacter and cytokine assays

The quantification of campylobacters provided the biggest challenge in this project, due to both specificity and sensitivity issues. Real-time PCR is a very sensitive technique, therefore, even minor primer dimer production led to alteration of the results. Publication searches resulted in a number of possible primers for universal campylobacter, *C. rectus* and *C. concisus* quantification. These primers (Table 3.1) were

tested using standard PCR (Section 3.2.7) with culture collection *Campylobacter* species (Table 3.2) and additional genera and species isolated from the human gastrointestinal tract (*Strep. salivarius*, *Staph. aureus*, *L. acidophilus*, *Bif. longum*, *F. nucleatum* CCUG 53249, *H. pylori*, *V. parvula*, *B. vulgatus*, *Prev. tannerae*, *Pept. anaerobius*, *E. coli*, *Ent. faecalis*) for specificity.

Table 3.1: *Details of primer pairs tested for campylobacter PCR assays, results of their use, with reasons for insufficiency.*

Primer	Sequence	Reference	Reason
C412F	GGA TGA CAC TTT TCG GAG C	Linton *et*	Primer dimer,
C1228R	CAT TGT AGC ACG TGT GTC	*al.*, 1996	multiple bands
CampF2	CAC GTG CTA CAA TGG CAT AT	Lund *et al.*,	Primer dimer
CampR2	GGC TTC ATG CTC TCG AGT T	2004	
Cuc1F	GCC CTA CAC TAG AGG ACA AC	This study	Multiple bands
Cuc1R	GAT AAT CCG CCT ACG CGT CC		
Cuc1F	GCC CTA CAC TAG AGG ACA AC	This study	Picks up other
Cuc3R	GAT AAT CCG CCT ACG CGT CC		genera
CampF	GGA TGA CAC TTT TCG GAG	Rinttilä *et*	Primer dimer
CampR	AAT TCC ATC TGC CTC TCC	*al.* 2004	
Camp*RpoB*F	CCC ACT ACG GCA GAA TTT GT	This study	Very small primer
Camp*RpoB*R	AGG AGC TTC AAC AAA GCC AA		dimer
C. conc 86F	AGC GGG CCT AAC AAG AGT TAT TAC A	Kawasaki	Primer dimer
C. conc 302R	TGT AAG CAC GTC AAA AAC CAT CTT T	*et al.*, 2008	
Pcisus1	GAG CTT GTG GTA AAG A	Matsheka	Multiple bands
Pcisus6	CCC GTT TGA TAG GCG ATA G	*et al.*, 2001	
Crec415F	TTT CGG AGC GTA AAC TCC TTT TC	Ashimoto	Multiple bands
Crec1012R	TTT CTG CAA GCA GAC ACT CTT	*et al.*, 1996	and primer dimer

Table 3.1 identifies the main primers tested and the results of the assays, indicating reasons for discard, such as primer-dimer production. The primer set designed by Rinttilä *et al.* (2004) offered the best detection, allowing for universal amplification of all tested campylobacters. This primer set was employed for PCR and sequencing of suspected campylobacters isolated by traditional culture (Chapter 2), however, under real-time PCR conditions, unsuitable levels of primer dimer were produced. The company Primer Design Ltd (Southampton, UK) provided sponsorship to develop a new, highly specific and sensitive primer, for detection of the *Campylobacter* genus.

Throughout months of consultancy, a primer pair very similar to that of Rinttilä *et al.* (2004) was designed, however, upon real-time PCR, this primer set still resulted in a low level of primer dimer product. A search for short sequences to amplify DNA from genes other than 16S rRNA was also performed, including the heat shock protein *groEL*, Type II topoisomerase *gyraseB*, the enzyme *phospholipase A* and finally the β-subunit of RNA polymerase, *rpoB*. This final gene provided the best match upon PCR analysis, and was highly specific to the tested campylobacters (Table 3.2), with no amplification of other bacteria (listed above). Quantitative results were obtained for all biopsy samples utilizing these *rpoB* primers (Camp*RpoB*F/R, this study, Table 3.1) with real-time PCR. Although some primer dimer was still observed during real-time PCR, the levels were low (10^1 - 10^2 SQ mean), which is common for this technique. To eliminate doubt, all products were tested for amplicon size using agarose gel electrophoresis, with results only taken from those with a band of the correct size (112 bp).

The second component of this research was to investigate host responses in oesophageal tissue relating to carriage of these organisms. Problems were encountered with primer sets for cytokine analysis of these biopsy tissues. Development of assays for a selection of cytokines was proposed (TNF-α, IL-1α, IL-1β, IL-8, IL-18, IL-4, IL-6, IL-10, Interferon-γ, IL-10). However, due to time constraints, only those for TNF-α, IL-1β, IL-8 and IL-18 were performed with cDNA from the tissue samples. Problems were encountered with either primer design or the final assay, yet it is unclear whether this was due to unspecific primer sets, or issues with positive samples taken from blood donors, including the methods for extraction.

3.1.3 Cytokine analysis in oesophageal disease

The induction of host responses due to infection was discussed in the Introduction (Sections 1.2.8 and 1.5.6). Interleukin-8 is a well studied cytokine in the immune response to BO and its development to ADC. Research has revealed an increased IL-8 expression in GORD patients compared with BO and non-inflamed squamous tissue (Fitzgerald *et al.*, 2002b), while Isomoto *et al.* (2003, 2007) discovered an increase in this cytokine with disease severity, and a correlation between increased expression and recurrence of oesophagitis. It is possible that this cytokine could be employed as a marker for disease severity and potential disease relapse. A further study by Fitzgerald *et al.* (2002a) investigated the response of Il-1β to oesophageal inflammation. They exposed patient samples to a bile acid cocktail (pH 4), finding increased expression after a pulsed, rather than continuous exposure, providing insight into the pathological differences between patients with varying patterns of reflux episodes. IL-1β is involved in the cellular activities of proliferation and apoptosis, and can additionally induce COX-2 production, contributing to inflammatory pain hypersensitivity (Samad *et al.*, 2001; Binshtok *et al.*, 2008). Conversely, Isomoto and co-workers found no significant difference in IL-1β levels between GORD and control subjects. However, expression of this cytokine correlated with IL-8 production, both of which, together with TNF-α, are regulated by NF-kappa B pathways, also found to be activated in these GORD patients (Isomoto *et al.*, 2003). A study investigating levels of IL-8 and IL-1β found higher levels in NF-kappa B positive compared with negative patients, being most significant in those with ADC. Additionally, expression of IL-8 and IL-1β increased with grade of GORD, presence of dysplasia and stage of ADC (O'Riordan *et al.*, 2005).

The similarity between gastric and oesophageal ADC has been discussed throughout this study, with comparable profiles for host responses. A study by Thong-Ngam and co-workers recruited 68 patients with either gastric cancer or gastric ulcers, employing ELISA to measure a range of immune responses (Thong-Ngam *et al.*, 2006). The expression of IL-18 was increased significantly in ADC vs. gastric ulcer samples. IL-18, formally known as IFN-γ inducing factor, stimulates both innate and adaptive responses (Th1 and Th2), inducing NK cell activity and apoptosis, and inhibiting angiogenesis in tumour cells. However, inappropriate production of this cytokine is thought to influence the clinical outcome of ADC, being involved in its pathogenesis (Lebel-Binay *et al.*, 2000). A significant difference in the protein levels of IL-18 was found in ADC patients compared with those with gastric ulcers; IL-18 might therefore be used as a diagnostic marker (Thong-Ngam *et al.*, 2006). There is no published research investigating the role of IL-18 in oesophageal adenocarcinoma, although Diakowska *et al.* (2006) used blood samples from 41 patients with oesophageal squamous cell carcinoma to measure serum levels of this cytokine. A correlation was observed between the expression of this protein and stage of disease, with significantly higher levels in stage IV compared with stage II and III patient cohorts. Consequently, if similar associations are found with oesophageal ADC, expression of this cytokine may be used for prognosis as well as diagnosis.

Chapter 2 of this study investigated the presence of viable organisms colonising the mucosa throughout various stages of oesophageal disease. The presence of campylobacters in GORD, BO and ADC is of great interest, and warrants further

research. A number of studies have investigated the inflammatory response to campylobacter, with induction of a number of cytokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8), triggered by cytolethal distending toxin (CDT), LPS, flagellins and their related secreted proteins (van Putten *et al.*, 2009). These cytokines recruit neutrophils and monocytes to the site of infection, and result in tissue damage. The induction of IL-8 by campylobacters when introduced to intestinal epithelial cell lines shows that *C. jejuni* and *C. coli* increase expression of this cytokine within 4 hours, with protein detection at 24 hours (MacCallum *et al.*, 2006; Borrmann *et al.*, 2007). The study by MacCallum and co-workers, used cell culture and human tissue recovered during surgery, to investigate the differences in response of these intestinal cell types. Although similar patterns of induction occurred after infection with *C. jejuni*, increased levels of IL-8 were found with human tissue, which was likely due to the full range of inflammatory cell types present and their ability to cross-talk. Interestingly, they also identified a difference in the response dependent on the anatomical site from which tissue was removed, proving the importance of primary tissue in understanding the bacteria-host interactions during health and disease.

Hence, research reported here aimed to identify the key organisms present in the oesophagus of patients in health and disease, and the possible effect of these mucosal populations on the host immune response, employing molecular real-time PCR technologies.

3.2 Materials and Methods

3.2.1 DNA extraction from bacterial isolates for assay development

Bacterial cultures were grown on Wilkins-Chalgren agar (Oxoid) for DNA extraction. Cells were swabbed into PBS and spun down to form a pellet, which was resuspended with 450 µl of molecular grade water (VWR Ltd., Leicestershire, UK) and 50 µl lysozyme (50 mg ml^{-1}) before incubating at 37^{o}C for 30 min. To this suspension, 25 µl Proteinase K (Qiagen kit), 50 µl 20% SDS and 500 µl molecular-grade water was added, together with 350 mg 0.1 mm sterile glass beads. Two bead-beating steps (Beadbeater-8, Biospec, Bartlesville, OK, USA) of 2 min with incubation at 60^{o}C for 10 min between, allowed release of the DNA into the supernatant from the lysed cells. The cell debris was removed by centrifugation (3 min, 5000 *g*) before purification, washing and elution of the DNA in a Qiagen mini-column (DNeasy Blood and Tissue Kit, Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions.

3.2.2 RNA extraction from peripheral blood

Blood was removed from the donor and mixed with a wash buffer (RPMI 1640, penicillin/streptomycin, HEPES buffer (all Invitrogen, UK) and Ficoll Paque (GE Health Systems, VWR, UK)). This suspension was centrifuged at 2600 rpm for 17 min (18^{o}C), before removing the white blood cells into fresh wash buffer, and spinning at 1750 rpm for 10 min. Wash buffer was then removed and the white blood cells shaken to break the pellet. The cells were kept on ice for 5 min before disruption with 350 µl lysis buffer (RTL buffer and mercaptoethanol). Lysates were poured into Qiashredder

columns (RNeasy Tissue Kit, Qiagen Ltd., West Sussex, UK) and the RNA extracted, washed and eluted as per the manufacturers instructions.

3.2.3 Reverse transcription PCR (RT-PCR)

Reverse transcription was performed with RNA, to produce more stable cDNA for use in PCR and real-time PCR. mRNA (Section 3.2.2) underwent a thermal cycle of 42^{o}C for 90 min followed by 95^{o}C for 5 min. Reagents and protocol used were provided by Primer Design Ltd. (Southampton, UK).

3.2.4 Primer development

Primer sets were designed, or further optimised, to target the small 16S rRNA (or *rpoB* for campylobacters) gene subunit of a select range of bacteria and cytokines for use in real-time PCR. Bacterial primers were selected based on knowledge obtained from previous culture and molecular-based studies of oesophageal microbiota composition in both health and disease (Pei *et al.*, 2004, 2005; Osias *et al.*, 2005; Macfarlane *et al.*, 2007; this investigation). Primer pairs for these assays are shown in Table 3.3. Primer sets for specific species were designed having aligned 16S rRNA gene sequences to highlight variations.

Lyophilised primers were purchased from Invitrogen. To prepare for PCR, they were dissolved in 300 μl PCR water (molecular biology grade water, BDH, VWR, Lutterworth, UK) and left on ice for 20 min, before being mixed and diluted 1:100 to determine concentration spectrophotometrically. The spectrophotometer was set to

measure absorbance at 260 nm for DNA. DNA concentrations were then measured using the following equation:

$$\left(\frac{A_{260} \times 20}{\text{Number of nucleotides in the primer} \times 0.33} \right) \times \text{dilution factor (100)}$$

Equation 3.1: *Measurement of primer concentration*

This equation is designed based on the knowledge that one unit of absorbance at 260 nm is equal to 20 $\mu g\ \mu l^{-1}$ DNA nucleotides, and that one nucleotide has a molecular weight of 0.33 $gmol^{-1}$, giving the final concentration in μM. Once the concentrations of the primers were known, they were prepared into 10 μM aliquots for use in the PCR reactions. All primers were stored at -80^{o}C.

3.2.5 Campylobacter assays

Five *Campylobacter* species were purchased from CCUG (Culture Collections, University of Goteborg, Sweden), with another two isolated from patient studies. The accession numbers for these species are provided in Table 3.2. The CCUG cultures were provided in their lyophilised form in ampoules. Upon delivery they were reconstituted with Wilkins-Chalgren broth warmed to 37^{o}C and spread on multiple plates of both WC blood agar and Columbia Blood supplemented with formate, fumarate, succinate and nitrate (1 g L^{-1} each) before storing for 3 days at 37^{o}C anaerobically and microaerophilically.

Table 3.2: *Accession numbers for different campylobacters (CCUG) required for universal campylobacter primer development.*

Campylobacter **species**	**Accession number/origin**
Campylobacter concisus	CCUG 34767
Campylobacter fetus	CCUG 41521
Campylobacter coli	CCUG 36766
Campylobacter lari	CCUG 22395
Campylobacter hyalointestinalis	CCUG 26151
Campylobacter jejuni	Barrett's oesophagus patient
Campylobacter rectus	Barrett's oesophagus patient

All bacteria were sub-cultured several times and observed microscopically before storage in freezing medium (WC broth, 2% mucin and 10% glycerol). Additionally, eubacterial primers (Table 3.3) were used for sequencing of the 16S rRNA, confirming their taxonomy. Section 3.1.2 gives full information on the experiments and design work for a highly specific and sensitive assay for whole campylobacter genus, with a primer pair for amplification of the *rpoB* gene being the final assay of choice.

3.2.6 Attaining optimum temperatures for PCR

Bacterial DNA was subjected to PCR (section 3.2.7) in an Eppendorf Mastercycler gradient machine. This allowed duplicate samples to be tested at a range of different temperatures. These samples had a standard protocol of 95^{o}C denaturation and 72^{o}C elongation steps, however, the annealing stage had temperatures ranging from 54^{o}C to 65^{o}C, with increments of 0.5^{o}C - 1^{o}C. When the PCR products were run using gel electrophoresis, the optimum temperature (Table 3.3) was identified as the assay where most product was present, and after which none was visible, due to primer denaturation.

***Table 3.3**: Target groups and sequences of PCR primers for bacterial amplification.*

Primer set	Target group	Sequence	Anneal temp. (°C)	Product size (bp)	Reference
GapDH 107F	Glyceraldehyde-3-phosphate	GGA AGG TGA AGG TCG GAG TC	56	183	Furrie *et al.* 2005b
GapDH 290R	dehydrogenase	TCA GCC TTG ACG GTG CCA TG			
Uni 330F	All eubacteria	ACT CCT ACG GGA GGC AGC AGT	58	200	Nadkarni *et al.* 2002
Uni 530R		GTA TTA CCG CGG CTG CTG GCA C			
Bif 8F	*Bifidobacterium* genus	AGG GTT CGA TTC TGG CTC AG	62	156	Kok *et al.* 1996
Bif 164R		CAT CCG GCA TTA CCA CCC			
Lact368F	*Lactobacillus* genus	AGC AGT AGG GAA TCT TCC A	58	341	Walter *et al.* 2001
Lact693R		CAC CGC TAC ACA TGG AG			Heilig *et al.* 2002
Bac 605F	*Bacteroides* genus	GTC AGT TGT GAA AGT TTG C	55	127	Modified from Bernhard
Bac 732R		CAA TCG GAG TTC TTC GTG			and Field, 2000
Staph961F	*Staphylococcus* genus	CTT ACC AAA TCT TGA CAT CCT TTG AC	62	207	Smith *et al.* (unpublished
Staph1167R		CCA CCT TCC TCC GGT TTG TCA CC			data)
CampRpoBF	*Campylobacter* genus (*rpoB*)	CCC ACT ACG GCA GAA TTT GT	59	112	This study
CampRpoBR		AGG AGC TTC AAC AAA GCC AA			
Camp405F	*Campylobacter* genus	GGA TGA CAC TTT TCG GAG	62	246	Rinttilä *et al.*, 2004
Camp633R		AAT TCC ATC TGC CTC TCC			
Fuso824F	*Fusobacterium* genus	CTA ACG CGA TAA GTA ATC	58	273	Rinttilä *et al.* 2004
Fuso1079R		TGG TAA CAT ACG AAA GGG			
Veill 58F	*Veillonella* genus	ATC AAC CTG CCC TTC AGA	62	343	Rinttilä *et al.* 2004
Veill 401R		CGT CCC GAT TAA CAG AGC TT			
Hpylori 402F	*Helicobacter pylori*	GAA GAT AAT GAC GGT ATC TAA C	58	139	Rinttilä *et al.* 2004
Hpylori 520R		ATT TCC ACC TGA CTG ACT AT			
C. conc 86F	*Campylobacter concisus*	AGC GGG CCT AAC AAG AGT TAT TAC A	62	217	Kawasaki *et al.* 2008
C. conc 302R		TGT AAG CAC GTC AAA AAC CAT CTT T			
Cjej 822F	*Campylobacter jejuni*	CAT CTC AGT AAT GCA GC	60	166	This study
Cjej 964R		ATA AGG TTC TTA GGA TAT CAA GC			
Crec 415F	*Campylobacter rectus*	TTT CGG AGC GTA AAC TCC TTT TC	62	598	Ashimoto *et al.* 1996
Crec 1012R		TTT CTG CAA GCA GAC ACT CTT			
TNFα 410F	Tumour Necrosis	TCT CGA ACC CCG AGT GAC AA	56	123	Furrie *et al.* 2005a
TNFα 514R	Factor-α	TAT CTC TCA GCT CCA CGC CA			
IL-8 102F	Interleukin 8	ATG ACT TCC AAG CTG GCC GTG GCT	60	291	Li *et al.* 2004
IL-8 369R		TCT CAG CCC TCT TAA AAA CTT CTC			
IL-18 966F	Interleukin 18	GAC GCA TGC CCT CAA TCC	58	105	Boeuf *et al.* 2005
IL-18 1051R		CTA GAGCGC AAT GGT GCA ATC			
IL-1betaF	Interleukin-1β	CCT GTC CTG CGT GTT GAA AGA	60	150	Boeuf *et al.* 2005
IL-1BetaR		GGG AAC TGG GCA GAC TCA AA			

3.2.7 Polymerase chain reaction

Standard amounts of DNA corresponding to target sequences were required for real-time PCR. Briefly, DNA and cDNA extracted from previously isolated bacteria or blood (cytokines), containing the target sequence, was amplified using each PCR primer pair (Table 3.3). Forty-nine μl of master mix was added to 1 μl of purified DNA. PCR amplification was undertaken on a Techne Thermal Cycler with the temperature profile as follows: an original 2 min cycle set at 42^{o}C, followed by 35 cycles; 1 min at 95^{o}C for incubation, annealing step for 1 min at the optimum temperature (dependent on the individual primer sets (Table 3.3)), elongation stage of 45 seconds at 72^{o}C. A final elongation stage of 10 min at 72^{o}C left the DNA ready for analysis by agarose gel electrophoresis. The amplicons were separated on 3% (wt/vol) agarose gel to identify products. The gel was made with 1.5 g of agarose heated with 50 ml 1 x TTE buffer, with 0.4 μl ethidium bromide added for visualisation of the bands with UV trans-illumination at 305 nm. The products were run at constant 90 V for 20 min before visualisation. Band sizes were determined using a DNA ladder (2-log DNA ladder, 0.1 - 10 kb, New England Biolabs, Beverly, MA).

3.2.8 Ligation, transformation and purification

The product of correct size and sequence was purified using the QIAquick PCR purification kit (Qiagen) and ligated into a vector using the pGEM-T® Easy Vector System I (Promega, Madison, USA) employing pGEM-T Easy Vector plasmids (Fig. 3.2). The vector, DNA and ligase were incubated for 30 min at room temperature, before transformation into JM109 competent *E. coli* cells (>10^{8} CFU μg^{-1}, Promega). The

bacteria were defrosted quickly on ice, before being added to sterile Falcon tubes with the above reaction mixture, and incubated for a further 30 min on ice. Transformants were heat shocked in a 42°C water bath for 45 sec before being placed back on ice for 2 min. Four hundred and fifty µl of SOC (Sambrook and Russell, 2001) also heated to 42°C, was then added to the transformed cells and incubated for 1 hour at 37°C in an orbital shaker (New Brunswick G24 Tabletop Incubator/Shaker) at 150 rpm.

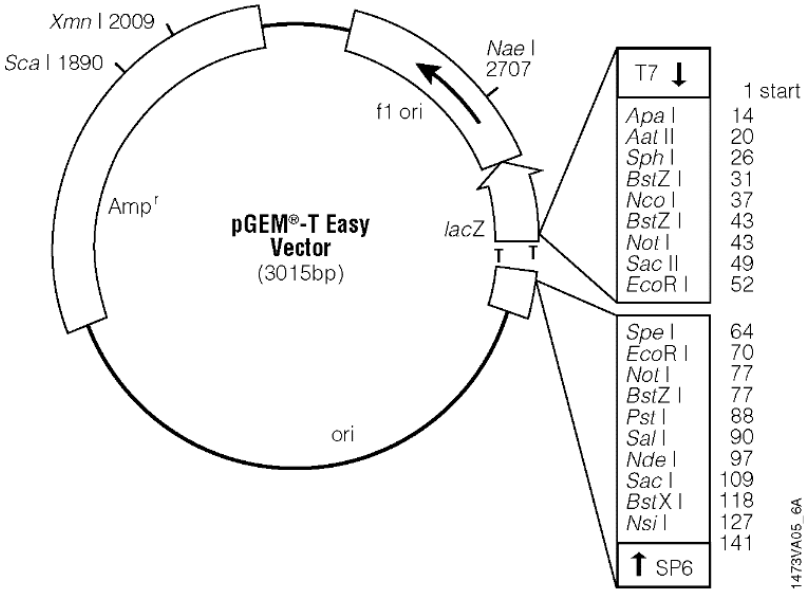


Fig. 3.2: *pGEM-T Easy Vector Plasmid*

The suspension was centrifuged (5 min, 1000 *g*) to collect the cultured cells, 350 µl of supernatant was removed, and the bacteria resuspended with remaining SOC prior to spreading on LB agar plates containing ampicillin for selection of transformed colonies. After overnight incubation, colonies contained the insert, because the pGEM®-T Easy Vector contains an ampicillin resistance gene for its selection. Colonies were picked and incubated in LB Broth with ampicillin overnight in the orbital shaker at 37°C. To check

the presence of insert in the plasmid, PCR was carried out with the overnight culture. Five hundred µl of the broth was centrifuged (5 min, 5000 *g*) to collect cultured cells. All supernatant was removed before resuspending the cells in 50 µl lysis buffer (1 ml 1% Triton-X-100, 2 ml 20 mM Tris-HCl and 74 mg 2 mM EDTA in 100 ml PCR water) and incubating on ice for 30 min to lyse the cells and release the plasmid. The suspension was then centrifuged at 20,000 *g* for 1 min, and 5 µl of this supernatant was removed for PCR using the appropriate primer pair (Table 3.3) for each clone, to check for the presence of the gene.

Plasmids were purified using the QIAprep Spin Miniprep Kit. Concentration of the plasmid preparation was determined by agarose gel electrophoresis, with known standards (New England Biolabs). The samples were diluted to 10^{10} molecules μl^{-1}, aliquoted and stored (-80^{o}C).

3.2.9 Patient recruitment

Biopsies were collected from patients as described in Chapter 2 (Section 2.2.1).

3.2.10 DNA extraction from patient biopsies

Extraction of bacterial DNA was carried out with a Qiagen DNeasy Blood and Tissue kit. Biopsy tissues were transferred aseptically from L-glutamine solution (GIBCO®, Invitrogen) into a sterile Eppendorf tube for storage at -80^{o}C. Frozen biopsies were suspended in 200 µl lysis buffer and 50 µl lysozyme (50 mg ml^{-1}) before incubating at 55^{o}C for 30 min. To mechanically disrupt the cells, 350 mg 0.1 mm glass beads (Biospec, Barlesville, OK, USA) were added to the biopsy suspension with 25 µl

Proteinase K, 200 µl Buffer ATL (both Qiagen kit) and 200 µl ethanol (96-100%) prior to bead beating for 2 min before and after incubation at 70^{o}C for 30 min. Centrifugation (3 min, 5000 *g*) removed all cell debris, allowing purification of DNA in the supernatant. DNA was adsorbed onto Qiagen mini-columns (DNeasy Blood and Tissue Kit) by centrifugation, and purified according to manufacturers instructions.

3.2.11 RNA extraction from biopsies

The liquid nitrogen technique for extraction of mRNA from biopsies was employed. A pestle and mortar were cooled with liquid nitrogen before adding biopsy tissue and grinding. The resultant powder was then poured into a Falcon tube, and after evaporation, 350 µl of lysis buffer (RTL buffer and mercaptoethanol) was added. The lysate was then poured onto a Qiashredder column and the RNA was extracted, washed and eluted as per the manufacturers instructions. cDNA was obtained using methods described in Section 3.2.3.

3.2.12 Quantitative real-time PCR

The appropriate plasmid preparation (Table 3.3) was diluted to give a standard curve of 10^{6}-10^{1} molecules µl^{-1} for all assays, except GapDH, which had a standard curve of 10^{8}-10^{1} molecules µl^{-1}. Real-time PCR was performed using an iCycler and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) using the optimum annealing temperature for each primer set (Table 3.3). Test samples (bacterial DNA and host cDNA) were added in duplicate at 2 µl per well in a 20 µl total reaction volume. Results are expressed as means of two separate experiments.

3.2.13 Statistical analysis

Statistical analysis was conducted using Prism Statistical Package (Section 2.2.5). Data were tested for Gaussian distribution using the Kolmogorov-Smirnov (K-S) test, with skewed results undergoing transformation before analysis. ANOVA (one-way analysis of variance) with Bartlett's test for equal variances, and Newman-Keuls post-hoc testing was carried out on data showing significance. Student's t-tests (two-tailed) were also carried out to measure variation between individual groups where no significance was seen with ANOVA, and for analysis of bacteria in male and female patients. Paired Student's t-tests were employed to analyse transformed results from ADC vs. squamous tissue from one individual. For cytokine analysis of the four groups, ANOVA was applied, while for the ADC and non-ADC tissue from matched patients, Wilcoxon Matched Signed Rank tests were performed for IL-8 and IL-1β, while paired data was insufficient for statistical analysis of TNF-α and IL-18. A P value of <0.05 was classed as statistically significant.

3.3 Results

3.3.1 Demographics

All patients were between the ages of 22 and 87, with mean ages of 58 to 63 in all patient cohorts, with a greater female to male ratio in control and GORD subjects (27:12 and 23:14, respectively). However, in BO and cancer patients, this shifted to a ratio of 31:14 and 25:9 males to females respectively, confirming previous evidence that men are at greater risk of BO and cancer (Lofdahl *et al.*, 2008). Twelve patients undergoing surgery for oesophageal resection volunteered to take part in this study. Samples were

taken during surgery from areas of cancerous and normal squamous tissue. Histological analysis revealed squamous cell carcinoma (SCC) in four of these patients, while the remaining eight were diagnosed with ADC. The remaining 22 ADC patient samples were acquired during upper gastrointestinal endoscopy. No significant variances were identified between ADC and SCC patient data, therefore, results from these two patient groups were analysed as a whole (cancer cohort).

Of 45 Barrett's patients, 53% (n = 24) had a hiatal hernia, nine IM, and two dysplasia. Interestingly, 34/45 BO patients were prescribed a PPI, whereas only 10/34 cancer patients were taking these drugs. Ten out of 39 controls and 26/37 GORD were taking PPIs, indicating that many of these cancer patients had not previously been diagnosed with oesophageal or gastric conditions. The cancer patients were also taking the least of any other drugs, including NSAID, cardiac or anti-depressants. Conversely, cancer patients had the highest alcohol consumption, with only eleven subjects stating an infrequent alcohol intake, compared to 20/39 controls, with remaining patients drinking between 1 and 70 units of alcohol per week (Table 3.4).

Table 3.4: *Clinical details of patients taking part in study for molecular analysis of oesophageal microbiotas.*

Characteristics	Controls (39)	GORD (37)	BO (45)	Cancer (34)
Sex, male:female	12:27	14:23	31:14	25:9
Age, years				
Range	22 – 87	24 – 83	31 – 84	40 – 80
Mean ± SEM	58.1 ± 2.3	63.3 ± 2.2	61.1 ± 1.4	63.5 ± 1.6
Body mass index				
<24 (range)	17 (18.1–23.2)	9 (17.3-24)	6(18.5-23.4)	15 (14.9 -24)
24.1-30	13 (24.1-29.8)	16(24.1-29.4)	25(24.1-29.7)	4(25.4-29.8)
>30	9 (30.3–39)	11(30.4-40.8)	14(31.2-39.4)	2(31.3-33.9)
Biopsy site, cm range				
Lower >28	39 (OGJ [a] - 30)	36 (42 – 30)	42 (42 – 30)	18 (43 - 29)
Middle 28-24		1 (28)	1 (26)	2 (28 – 25)
Upper <24				
GORD inflammation				
Grade A		14		
Grade B		12		
Grade C		2		
Grade D		2		
Pathology/Histology				
Hiatus hernia	4	12	24	1
IM[b]			9	
Dysplasia			2	
ADC:SCC[c]				30:4
Current treatments				
PPI	10	26	34	10
NSAIDS	7	12	14	2
Cardiac	13	19	15	3
Others	2	6	8	0
Probiotics	9	4	6	4
Smoker (ex smoker)	5 (3)	7 (5)	11	10 (2)
Alcohol (range)				
None	20	15	17	11
Beer	9 (1-100)	10 (2-24)	15 (2-28)	5 (7-50)
Wine	6 (1-14)	8 (2-20)	10 (2-20)	6 (1-70)
Spirits	6 (1-12)	2 (8)	4 (2-7)	3 (1-21)

[a] biopsies taken 5 cm above the OGJ. [b] Intestinal metaplasia. [c] Cancer patients diagnosed with adenocarcinoma or squamous cell carcinoma.

3.3.2 Real-time PCR analysis of oesophageal bacteria

Molecular analysis of bacterial abundance was carried out with whole DNA extracted from patient biopsies. Total eubacterial populations had a relatively stable spread in all patients, however, comparisons of the four means with ANOVA, found a significant difference, with $P = 0.0438$. Newman-Keuls post-hoc testing found the variation was attributable to differences in the means of control and ADC patients ($P < 0.05$) (Fig. 3.3). Analysis of individual bacterial genera indicated that the composition of these bacterial populations varied widely. Gaps in cell numbers existed in these patients signifying the need for a larger range of assays to be undertaken.

Figure 3.4 shows results from the bifidobacterium assay, levels of these bacteria were reduced in GORD patients before recovering towards normal in cancer. ANOVA did not show a significant variation between means in these patients. However, the Bartlett's test for equal variance highlighted a shift in the distribution of data ($P = 0.0163$). Bacteroides levels were variable throughout (Fig. 3.5), with statistically significant variation between all four groups ($P = 0.0338$). Fusobacteria populations were significantly reduced in GORD compared with normals ($P < 0.001$, Newman-Keuls) (Fig. 3.6). ANOVA gave a P value of 0.0001, indicating highly significant variation between the mean of all four patient groups.

Veillonella also showed a decrease in diseased patients compared with normals ($P < 0.0001$ (Fig. 3.7)). Distribution of data and therefore, levels of this species in each individual, were highly variable, indicated by the Bartlett's test ($P = 0.0005$).

Lactobacilli were similar in most patients, with a small number of outliers in each group (P = 0.0008, Bartlett's) (Fig. 3.8). Staphylococci (Fig. 3.9) varied significantly throughout patient cohorts, with a P value of 0.0003 for all four means (ANOVA) and P = 0.0043 (Bartlett's). Additionally, Newman-Keuls post-hoc testing revealed significant differences between controls and the three disease groups. In Fig. 3.10, *Helicobacter pylori* data indicates that prevalence was low in the majority of patients. In the control group there were 3 outliers with higher populations, however the others were similar to GORD and BO patients. The cancer group had the lowest *H. pylori* prevalence, in accordance with the theory of cancer protection from this species. ANOVA found a significant difference between all means ($P < 0.0001$), with statistically significant reductions between ADC and controls, GORD and BO ($P < 0.001$, 0.001, 0.01, respectively).

Real-time PCR results with primer sets amplifying the *rpoB* gene for the genus campylobacter are shown in Fig. 3.11, with expression from only five normals and three cancer subjects. Due to primer dimer products found in negative samples, results were only taken from samples with a positive band visualised on agarose gels. ANOVA did not show any significant differences in the mean bacterial population for each group. Populations of campylobacter were higher in BO patients than in GORD, with an almost significant difference in their means found with the Student's t-test (P = 0.0814). Although there were only three cancer patients, their prevalence was significantly higher compared to GORD (P = 0.0266); however, the quality of this analysis is limited by the sample size.

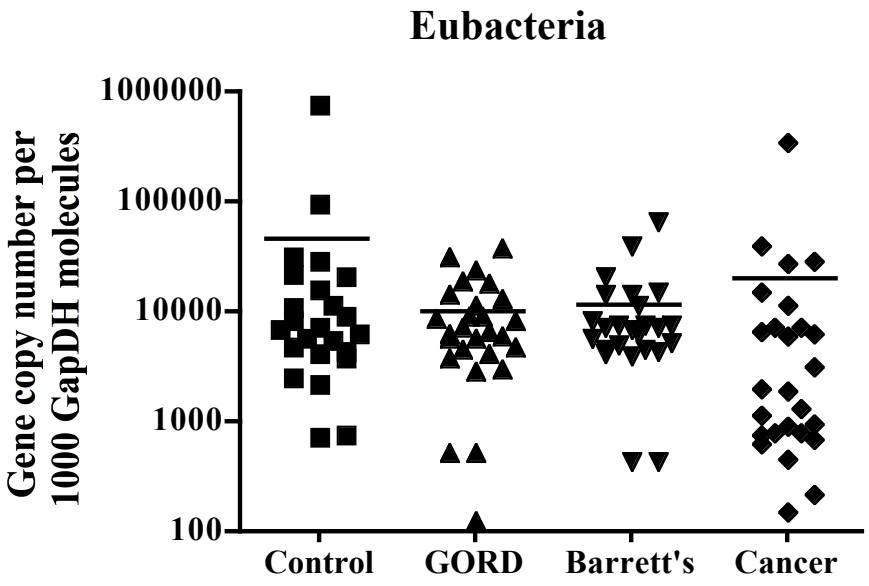


Fig. 3.3: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total eubacteria for each patient. Results were obtained for 24/39 controls, 27/37 GORD, 25/45 BO and 26/34 cancer patients. P = 0.0438, ADC vs. Control, P < 0.05.*

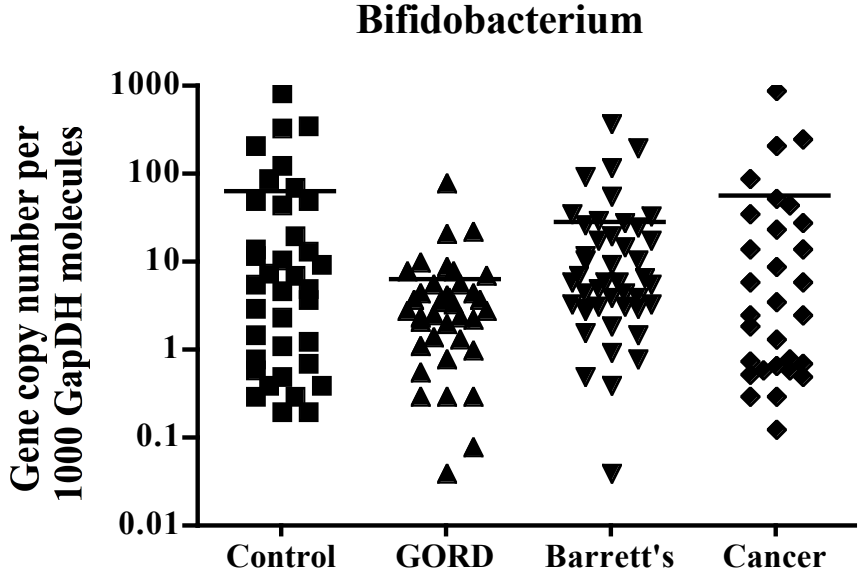


Fig 3.4: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total bifidobacteria for each patient. Results were obtained for 37/39 controls, 37/37 GORD, 45/45 BO and 30/34 cancer patients. GORD vs. BO and control (P = 0.003 and 0.0402 respectively, (t-test)).*

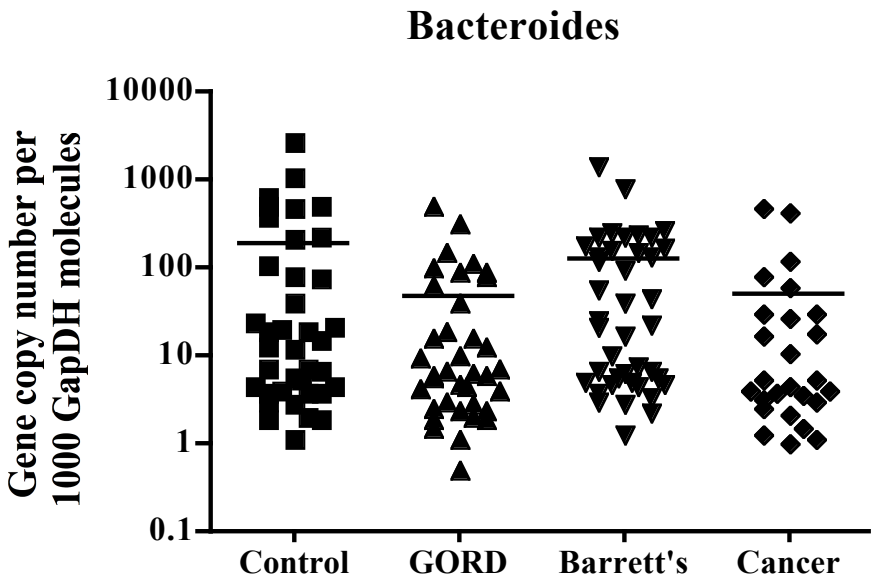


Fig. 3.5: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total bacteroides for each patient. Results were obtained for 38/39 controls, 37/37 GORD, 41/45 BO and 26/34 cancer patients. P = 0.0338 (ANOVA). Comparison of GORD vs. BO and ADC gave a P value of 0.0159 and 0.0202, respectively (t-test).*

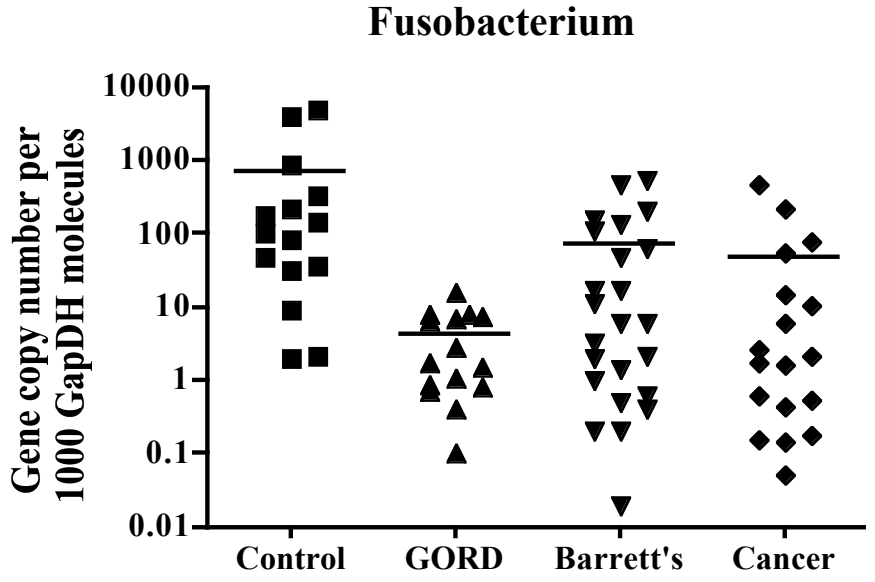


Fig. 3.6: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total fusobacteria for each patient. Results were obtained for 15/39 controls, 15/37 GORD, 24/45 BO and 18/34 cancer patients. Comparison of all four cohorts gave a P value of 0.0001, with control versus GORD, BO and cancer patients being highly significant (P < 0.01).*

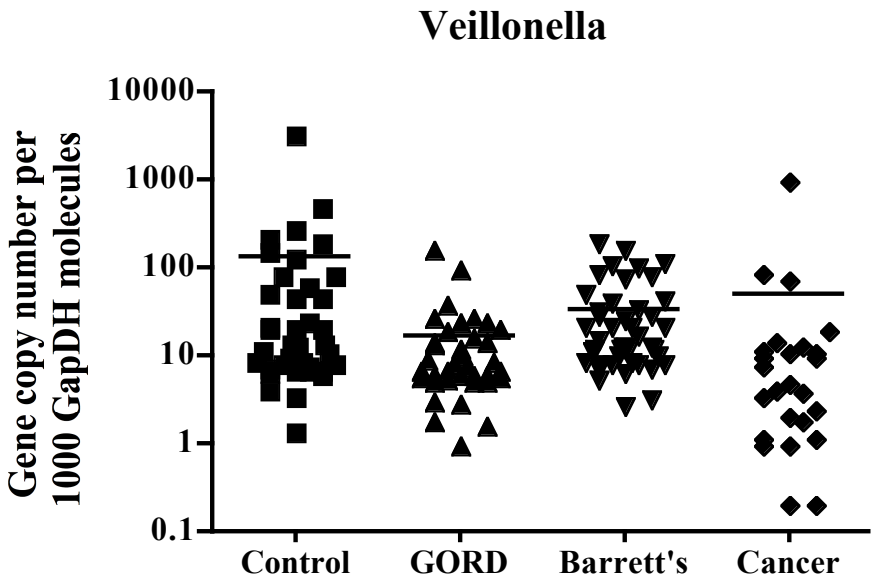


Fig. 3.7: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total veillonella for each patient. Results were obtained for 38/39 controls, 37/37 GORD, 45/45 BO and 25/34 cancer patients. Comparison of all four cohorts gave a P value of <0.0001, control and BO vs. ADC (P < 0.001), GORD vs. control and BO (P < 0.05).*

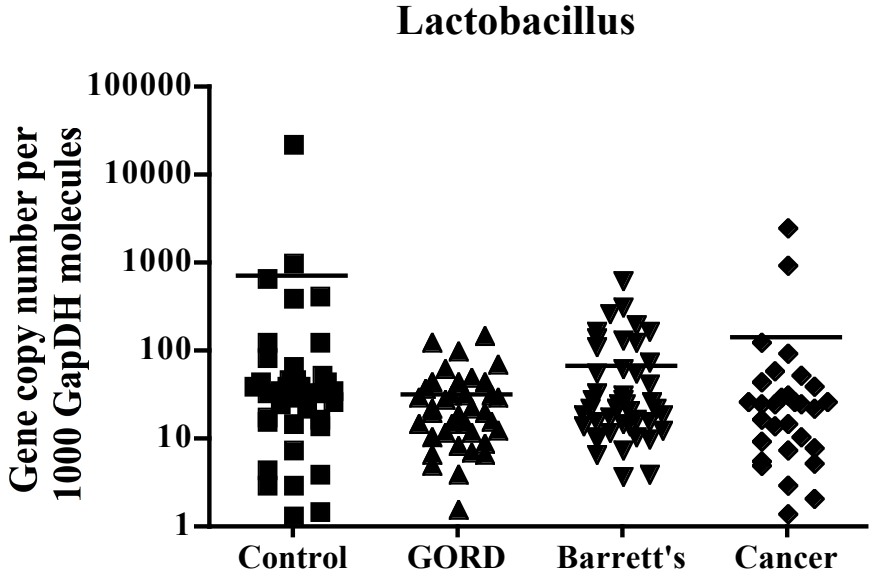


Fig. 3.8: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total lactobacillus for each patient. Results were obtained for 38/39 controls, 37/37 GORD, 45/45 BO and 30/34 cancer patients. Bartlett's test for equal variances (P = 0.0008), no other significant differences observed.*

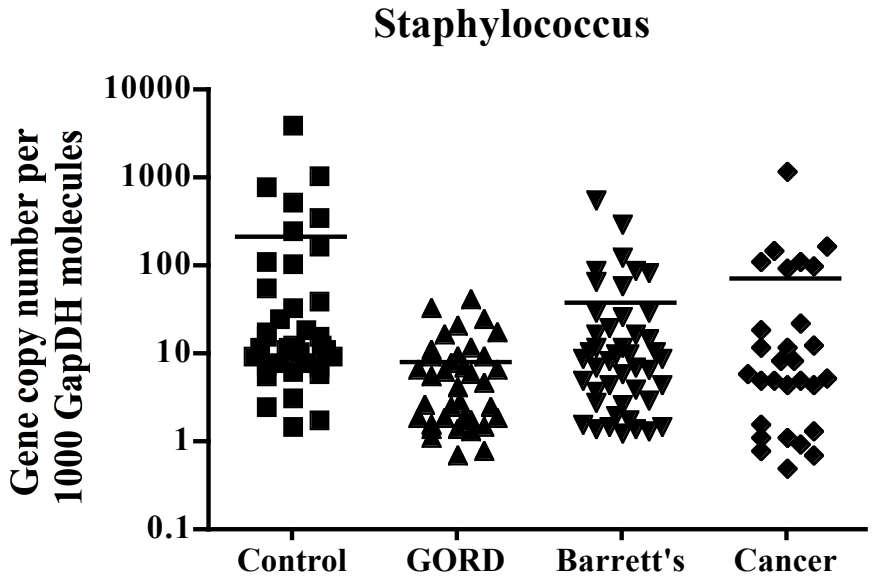


Fig. 3.9: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total staphylococcus for each patient. Results were obtained for 38/39 controls, 37/37 GORD, 45/45 BO and 29/34 cancer patients. Comparison of all four cohorts gave a P value of 0.0003 (ANOVA) and 0.0043 (Bartlett's). Control vs. GORD, BO and ADC ($P < 0.05$).*

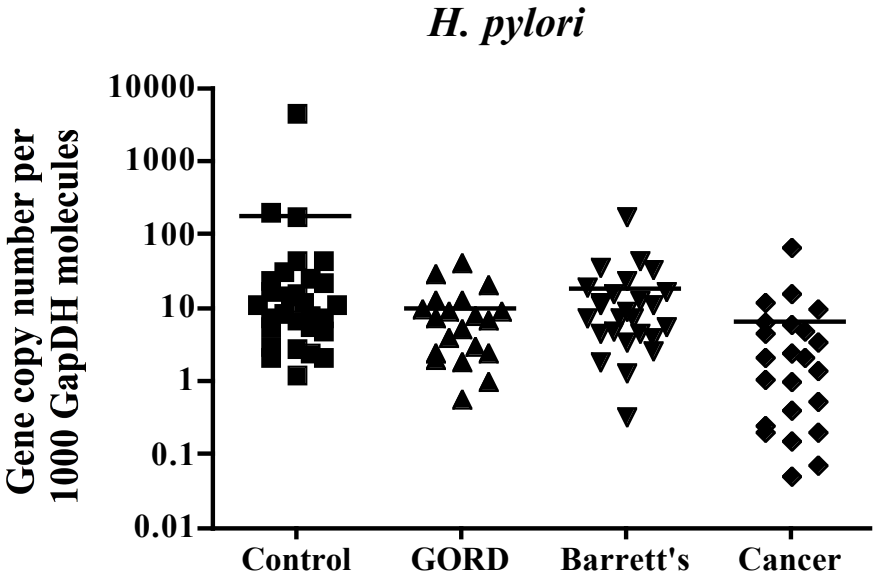


Fig. 3.10: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of H. pylori for each patient. Results were obtained for 29/39 controls, 20/37 GORD, 26/45 BO and 23/34 cancer patients. Comparison of all four cohorts gave a P value of 0.0001 (ANOVA) and 0.06 (Bartlett's). ADC vs. control, GORD and BO ($P < 0.01$).*

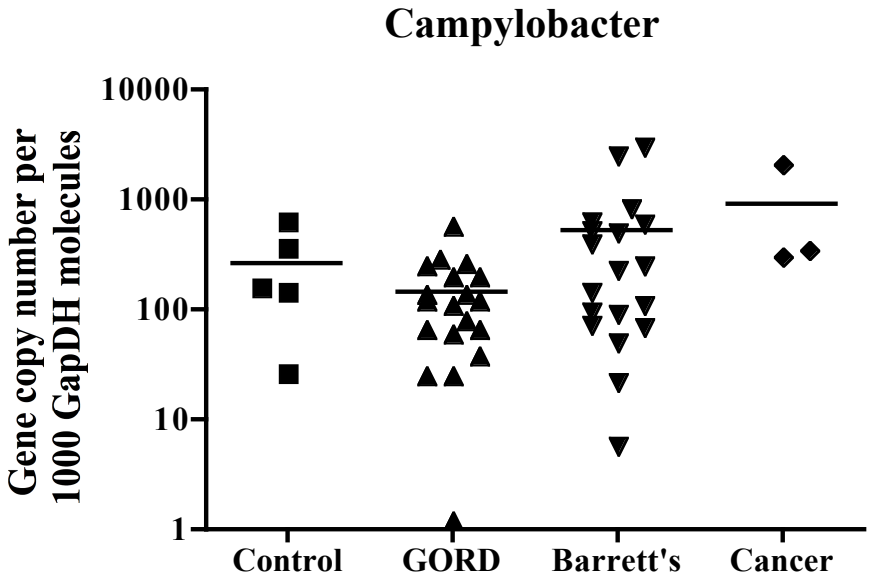


Fig. 3.11*: Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of total campylobacter for each patient. Results were obtained for 5/39 controls, 19/37 GORD, 19/45 BO and 3/34 cancer patients. Comparison of GORD vs. BO and cancer gave P values of 0.0814, and 0.0266, respectively (t-test).*

Analysis of microbiological results in patients prescribed a PPI compared with those not receiving PPI did not show any statistically significant differences. Interestingly, none of the five control patients with occurrence of campylobacter were taking a PPI. However, levels of campylobacter in the diseased patients were similar in both cohorts (Fig. 3.12).

Variations in bacterial colonisation of male and female patients were also analysed (Fig. 3.13). Eubacterial numbers were highest in males, with a significant difference between male and female control patients (P = 0.0355). Statistically significant differences between the sexes were also found in control patients with colonisation by veillonellas (P = 0.0066). While fusobacteria were more prevalent in female subjects than males in patients with both GORD and cancer (P = 0.0093, and 0.0167, respectively).

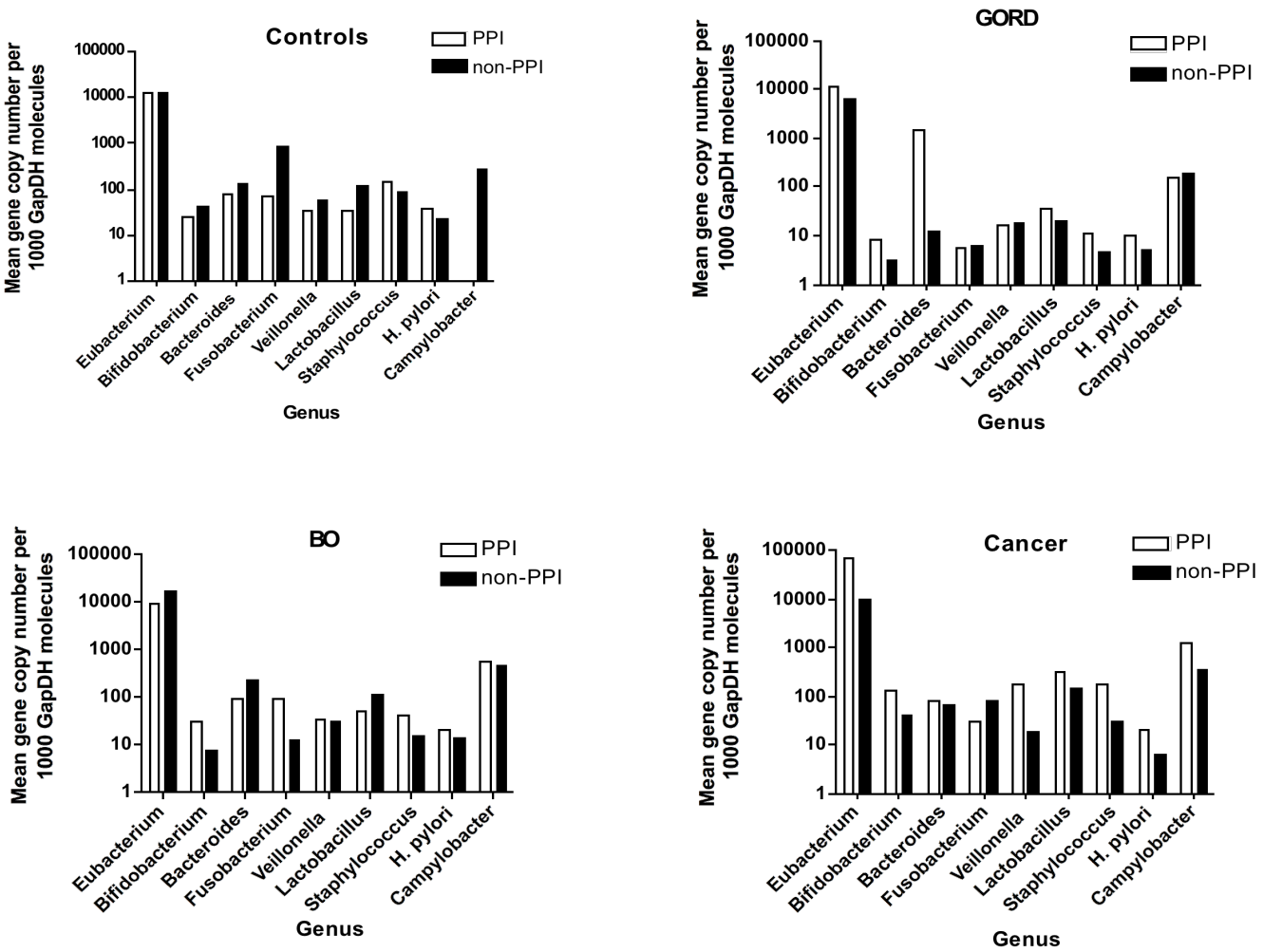


Fig. 3.12: *Gene copy number per 1000 gapDH molecules of biopsy for each bacterial genus isolated from patients prescribed PPIs compared with those not on PPI therapy. No significant differences were found between these patient groups.*

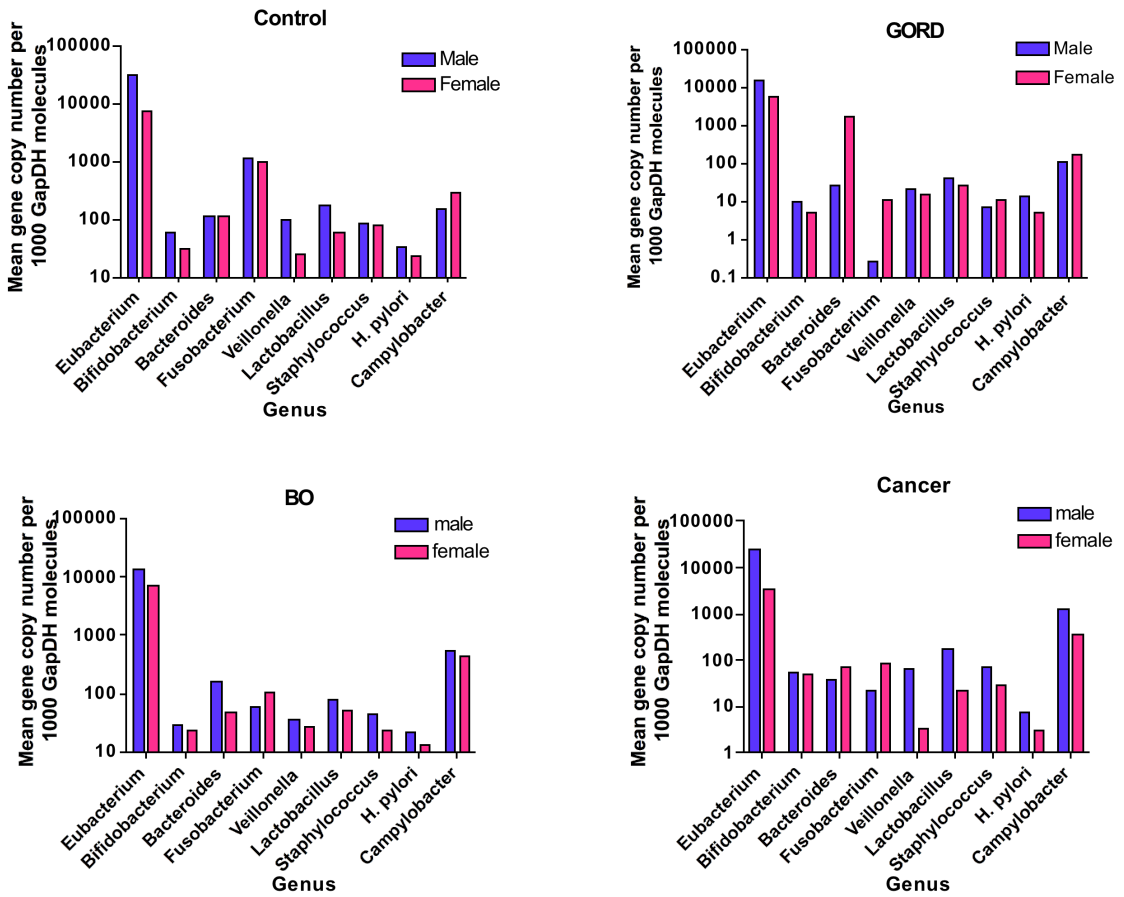


Fig. 3.13: *Gene copy number per 1000 gapDH molecules of biopsy for each bacterial genus isolated from males and females. Comparison of Eubacteria (t-test), Bacteroides and Veillonella between these sexes in control patients gave P values of 0.0092, 0.0497, and, 0.0199 respectively. Eubacterial (t-test) counts in GORD patients had a significant difference of P=0.001 between male and female patients. Comparison of fusobacterium in cancer patients gave a P value of 0.0186. Mann-Whitney U test applied unless stated.*

Slightly higher levels of campylobacter were detected in males with BO, and especially ADC, with the opposite seen in control and GORD (no statistically significant differences).

Cancerous and normal matched tissue from twenty-six patients was also analysed to compare intra-individual variances in the microbiota dependent only on tissue morphology. Data was not normally distributed and was thus transformed, allowing results to be analysed using a paired Student's t-test. The eubacterial assay indicated that there was no expression in a number of the non-cancerous tissues. Limited statistical analysis revealed a P value of 0.0137 (Fig. 3.14), with higher prevalence of bacteria seen in the matched normal squamous tissue compared with that at the cancerous site. Bifidobacteria had similar detection values in both tissues, although, levels were generally diminished in matched diseased tissue (P = 0.0091) (Fig. 3.15). Staphylococcal (Fig. 3.16) prevalence was similar in all tissue types; however, population means were significantly higher in the matched non-cancerous tissue (P = 0.0001). Bacteroides (Fig. 3.17) were found in similar numbers throughout, however, the majority of patients revealed a decrease in colonisation of these species on cancerous tissue (P = 0.0083). Veillonella (Fig. 3.18) was found in higher numbers in the normal tissue (P = 0.0263). Similar results were obtained with *H. pylori* assays (Fig. 3.19), with higher mean values for normal tissue (P = 0.0005). In a number of patients, *H. pylori* was detected in either cancerous or non-cancerous tissue only. However, in all of the subjects where this species was found in both tissue types, the prevalence was increased in normal squamous tissue.

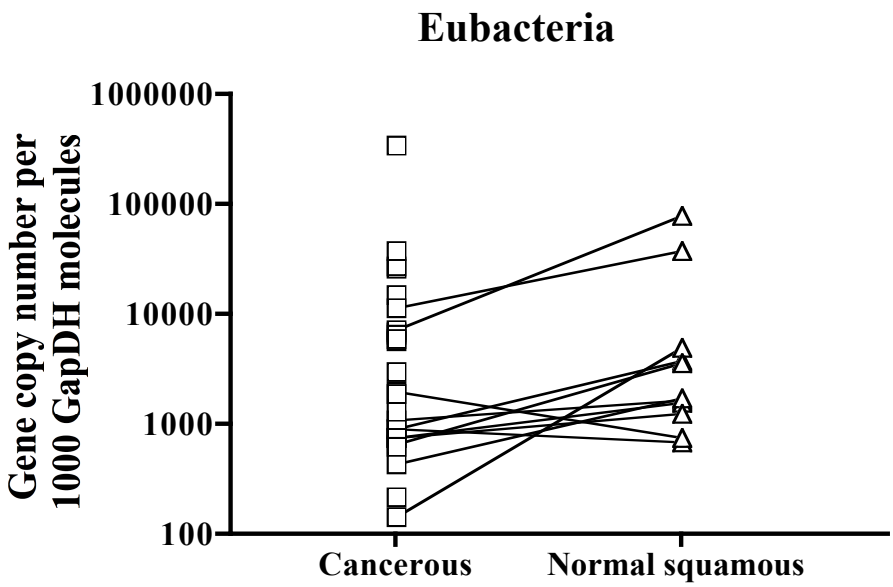


***Fig. 3.14**: Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of eubacteria for each patient. Results were obtained for 26 cancerous and 11 normal squamous tissues (P value 0.0137).*

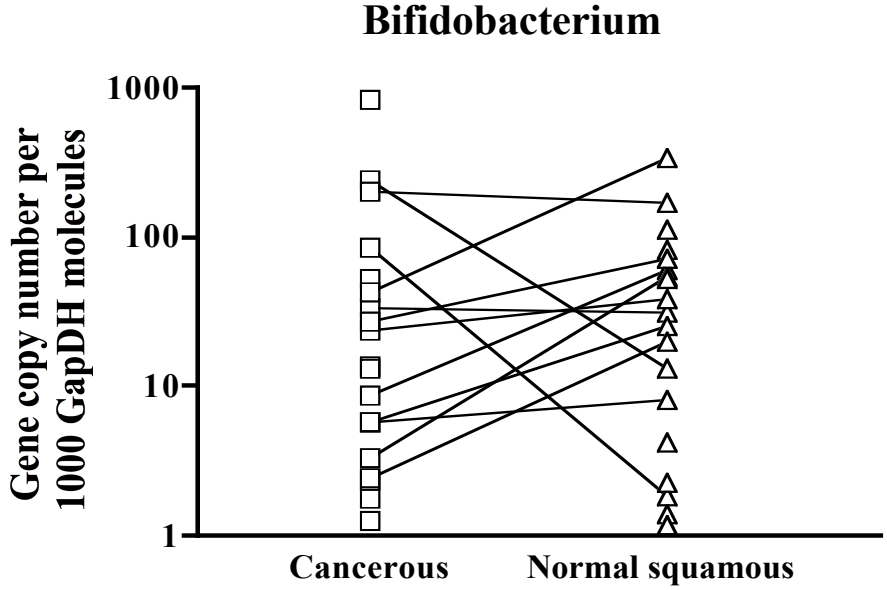


***Fig. 3.15**: Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of bifidobacteria for each patient. Results were obtained for 30 cancerous and 22 normal squamous tissues (P value 0.0091).*

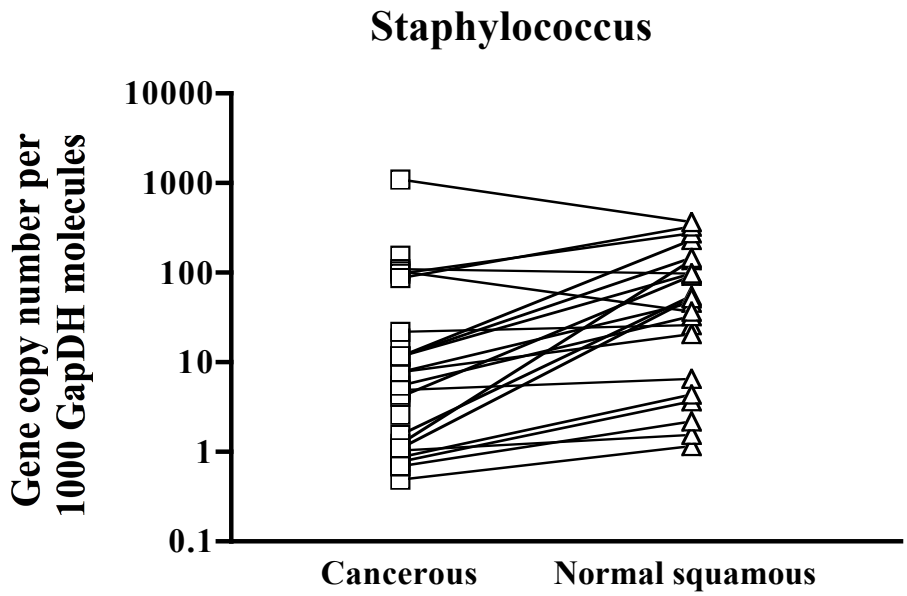


Fig. 3.16: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of staphylococcus for each patient. Results were obtained for 30 cancerous and 22 normal squamous tissues (P value 0.0001).*

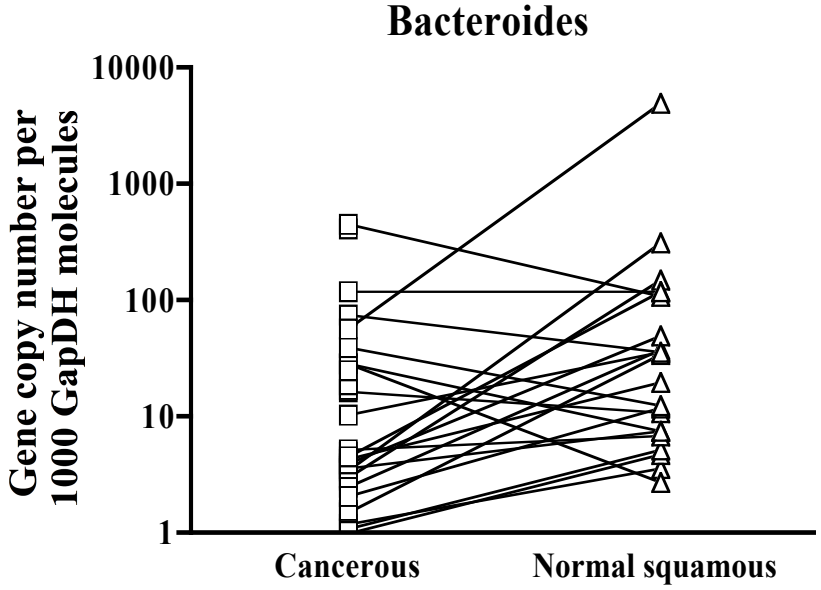


Fig. 3.17: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of bacteroides for each patient. Results were obtained for 30 cancerous and 22 normal squamous tissues (P value 0.0083)*

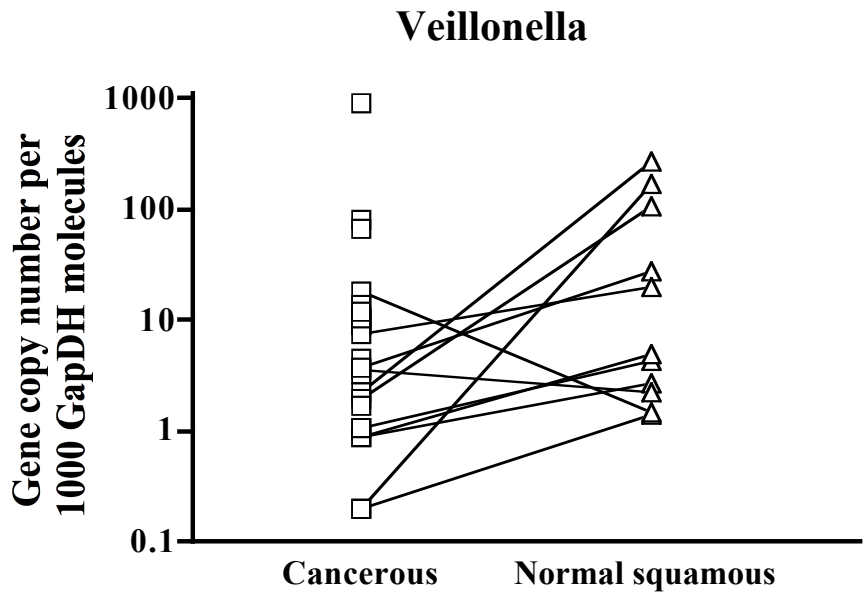


Fig. 3.18: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of veillonella for each patient. Results were obtained for 25 cancerous and 11 normal squamous tissues (P value 0.0263).*

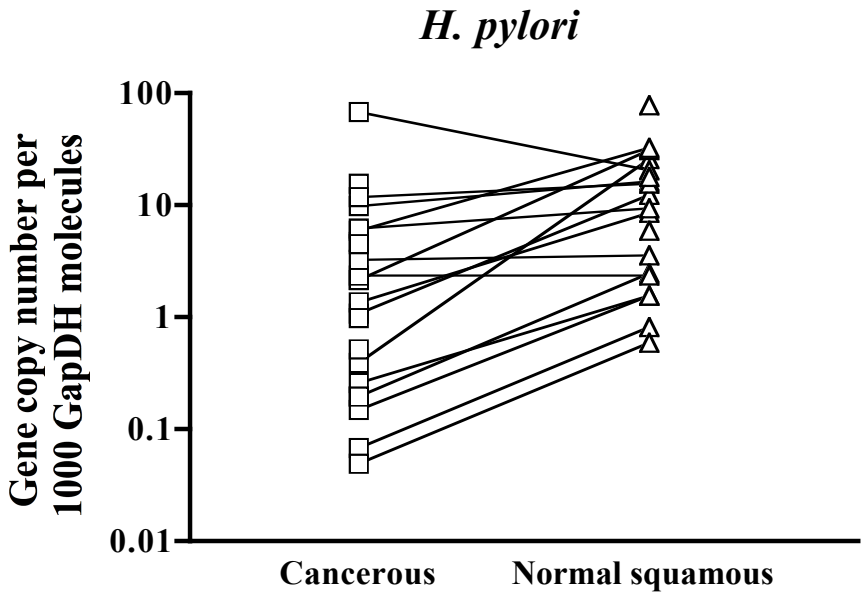


Fig. 3.19: *Gene copy number per 1000 GapDH molecules of biopsy showing individual counts of H. pylori for each patient. Results were obtained for 23 cancerous and 20 normal squamous tissue (P value 0.0005).*

3.3.3 Molecular analysis of cytokine expression

Analysis of a small number of cytokines (Section 3.1.2) previously associated with BO and ADC was performed to measure variations in host responses with disease progression. cDNA was extracted from patient biopsies and expression was measured against GapDH molecules. No statistical differences were found between mean cytokine levels in diseased groups compared with controls for any of the pro-inflammatory cytokines tested. The number of patients with expression of TNF-α were low compared with the other three assays, since there was no detection through real-time PCR for a number of the samples. Levels of mRNA expression for each cytokine were widespread through all patients, ranging from $1x10^{-5}$ to $1x10^{6}$ (Fig. 3.20 – Fig. 3.23).

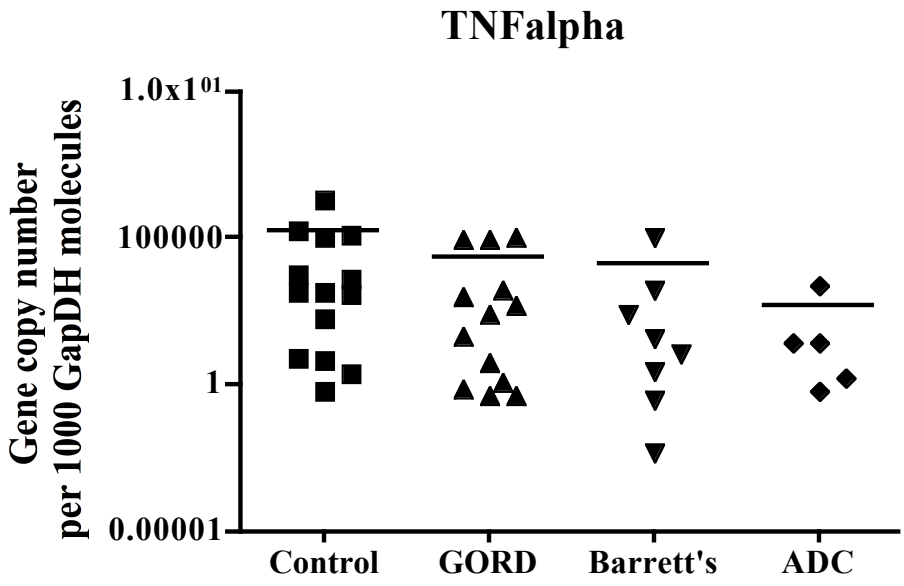


Fig. 3.20: *Gene copy number per 1000 GapDH molecules of biopsy showing individual mRNA expression for TNFalpha in each patient. Results were obtained for 14/39 controls, 13/37 GORD, 8/45 BO and 5/34 cancer patients. No Significant differences were observed.*

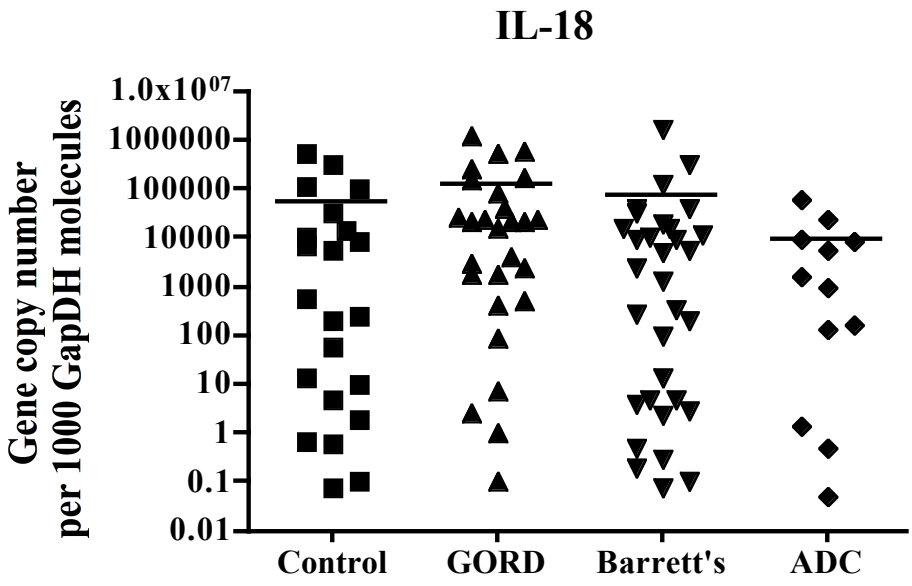


Fig. 3.21: *Gene copy number per 1000 GapDH molecules of biopsy showing individual mRNA expression for IL-18 in each patient. Results were obtained for 22/39 controls, 27/37 GORD, 32/45 BO and 12/34 cancer patients. No Significant differences were observed.*

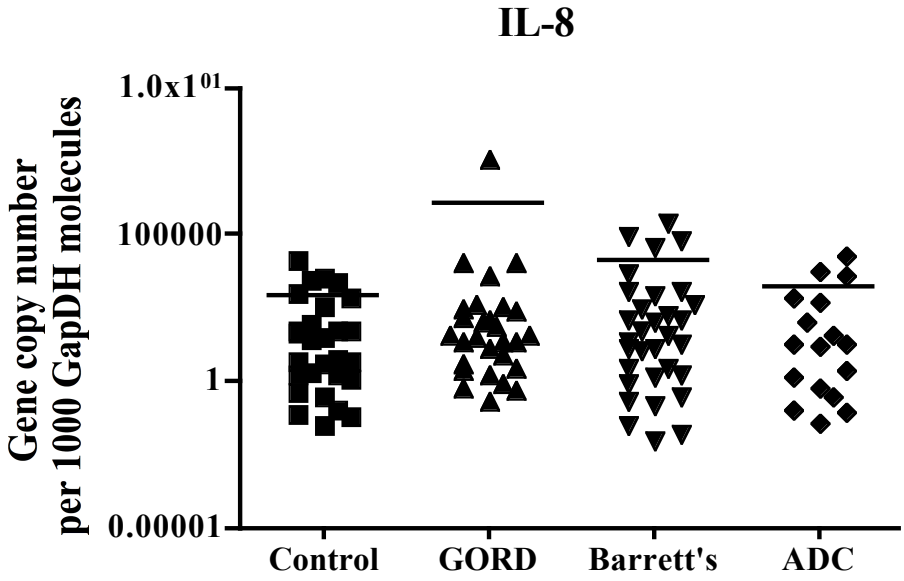


Fig. 3.22*: Gene copy number per 1000 GapDH molecules of biopsy showing individual mRNA expression for IL-8 in each patient. Results were obtained for 28/39 controls, 28/37 GORD, 32/45 BO and 17/34 cancer patients. No Significant differences were observed.*

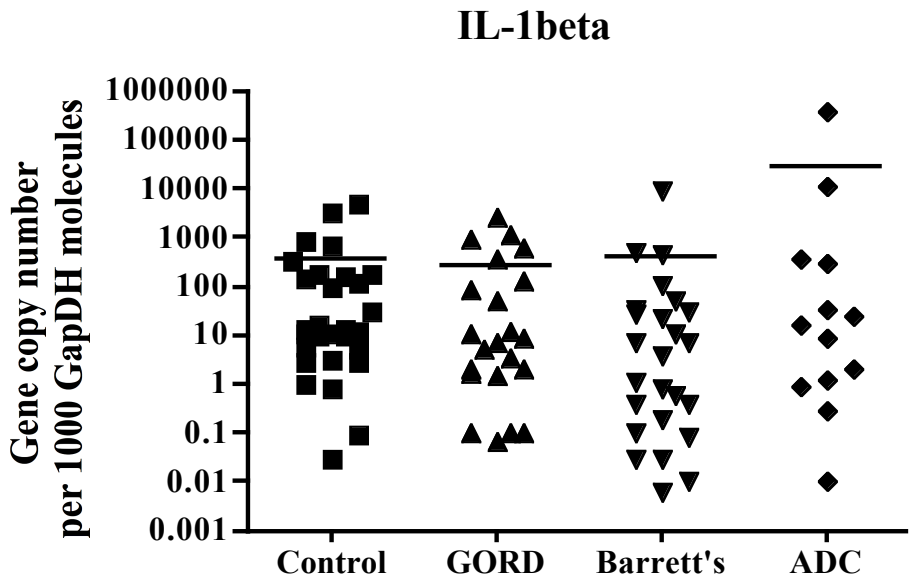


Fig. 3.23: *Gene copy number per 1000 GapDH molecules of biopsy showing individual mRNA expression for IL-1beta in each patient. Results were obtained for 29/39 controls, 22/37 GORD, 25/45 BO and 13/34 cancer patients. No Significant differences were observed.*

Cancerous and non-cancerous tissue from the same cancer patient was also analysed for variances in the cytokine response in these two sites. No significant differences were identified, possibly due to the minimal volume of data available, mainly from normal squamous matched tissue. This is likely attributable to either not receiving normal healthy tissue from the clinician carrying out the procedure, or, no expression was detected during real-time PCR analysis. Plots of these cytokine data are not shown.

3.4 Discussion

This chapter aimed to further characterise the oesophageal microbiota composition in patients with GORD, BO and cancer, compared to controls. The use of real-time PCR is a faster technique compared to culture, allowing a greater number of samples to be analysed quantitatively. Results from Chapter 2 did not identify a key organism that was present only in disease, and it was concluded that mucosal populations in the oesophagus shift to a more varied, Gram negative composition, which may result in disease progression.

This study represents the first investigations of the oesophageal microbiota in cancer, with 34 patients recruited for culture and molecular analysis. The demographic data confirms that the majority of patients who develop cancer are males (Lofdahl *et al.*, 2008). Interestingly, 50% of controls, 40% of GORD, and 38% of BO, stated that they did not drink alcohol, compared with 32% of cancer patients. This progressive increase in alcohol consumption with disease progression is interesting with data on alcohol and oesophageal ADC risk being inconclusive and contradictory (Pandeya *et al.*, 2009). The majority of these patients who did drink consumed only low levels, however, an ADC patient stated intake of 50 units of beer per week, while one SCC subject drank 70 units of wine per week. Alcohol consumption is a known major risk factor for SCC development, with polymorphisms in ALDH2 and ADH1B1, enzymes involved in alcohol metabolism (Lao-Sirieix *et al.*, 2010; Pandeya *et al.*, 2009).

Microbiological data from this part of the study corresponds with results from Chapter 2, indicating a shift in mucosal population composition during disease progression.

Information is limited due to the nature of real-time PCR, with data only collected for those bacteria chosen for analysis. Therefore, assays for the major genera identified in Chapter 2 were developed, however, this does not take into account individual variations and minor species. In contrast, characterisation of the oesophageal microbiota using PCR clone libraries (Pei *et al.*, 2004, 2005; Yang *et al.*, 2009), allows total bacterial DNA to be identified, therefore, all species present are detected. Nevertheless, as discussed previously, this technique has pitfalls. The DNA detected by real-time PCR may also have multiple origins and could provide false information. Due to the design of this study, using cultural results to design real-time PCR assays, it is hoped that these pitfalls have been avoided, providing quantitative results for viable bacteria colonising the oesophageal mucosa. There is a large gap in numbers between total eubacteria and individual bacterial genera in these patient cohorts. However, cultural and molecular analyses of the oesophageal microbiota identified streptococci as the predominant bacteria, comprising between 12% and 78% of the total community (Pei *et al.*, 2004, 2005; Yang *et al.*, 2009; Macfarlane *et al.*, 2007, this study). An assay for the genus *Streptococcus* could not be designed, with primers unable to detect all species in this genus. Additionally, assays for neisseria, actinomyces and rothia were not utilized in this research, which, from information in Chapter 2, account for up to 6%, 10% and 10%, respectively (Fig. 2.14 – 2.16). The lack of data for these four genera may account in large part for the discrepancy in the real-time PCR results.

Levels of *H. pylori* were low in all patients, but the organisms were detected in a greater percentage of controls compared with diseased tissues. This species was not recovered from any patients by culture (Chapter 2), which could be due to their low prevalence in

the oesophagus, being undetectable on culture medium. In control, GORD and BO patients this species had similar detection levels, clustering at 1-100 gene copy numbers per 1000 GapDH molecules. Conversely, in cancer patients, detection levels were distributed across a greater range, but at lower values. A statistically significant reduction in *H. pylori* prevalence was found in the disease cohorts compared with controls. This corresponds with previous studies describing cancer protection by this species, possibly due to presence of the cagA pathogenicity island. Jones *et al.* (2003) co-cultured this species with the ADC cell line, OE33, and normal oesophageal cells. *H. pylori* $cagA^+$ and $cagA^-$ species were added to these cell lines, intact and sonicated, to measure their ability to apoptose oesophageal cells. Adenocarcinoma cells underwent apoptosis at a higher rate than control cells, a process which was dependent on the presence of $cagA^+$ genes (Jones *et al.*, 2003). Results from this study may explain why *H. pylori* is protective against cancer, since its presence in the oesophagus of healthy patients prevents abnormal cells from continuing to survive, halting metaplastic progression to dysplasia.

Many bacteria in the genus *Bifidobacterium* are known probiotics, and there has been extensive research into their anti-inflammatory properties. The prevalence of this genus was reduced in GORD patients compared with controls, however, it increased with further disease progression. Results from cancerous and normal squamous matched tissues identified a reduction in the number of bifidobacteria in cancerous tissue. Although two patients had a marked increase in colonisation in their diseased tissue, 76% of these matched patients showed the reverse.

Kim *et al.* (2010) tested the effect of *B. lactis* on the inflammatory response of human colon adenocarcinoma cell cultures (HT-29), and in mice with induced colitis-associated cancer. This species down-regulated NF-kappa B pathways and inhibited NF-kappa B regulated genes in these intestinal epithelial cells and in *in vivo* models, reducing the number and size of tumours present in mice (Kim *et al.*, 2010). As discussed previously (Section 3.1.3), NF-kappa B regulates the expression of IL-8, IL-1β, and TNF-α, which are all extensively studied cytokines in oesophageal disease progression (Fitzgerald *et al.*, 2002a, 2002b; O'Riordan *et al.*, 2005).

The reduction in prevalence of bifidobacteria in GORD patients could allow further inflammation and disease progression. The occurrence of bifidobacteria in BO and cancer had a similar spread to that of controls, however, microbiome composition has much intra-individual variation, with this study highlighting these changes between healthy and diseased tissues in the oesophagus. Biopsies were only taken from the diseased tissues of GORD and BO patients, and therefore, the presence of this genus in non-diseased tissues may have been higher, with a reduction in those tissues which were inflamed or metaplastic, allowing for continued disease progression. Research into the use of probiotic therapy in oesophageal disease needs to be conducted, investigating their ability to reduce inflammation and inhibit disease progression.

The enteropathogen *Campylobacter*, has been identified as a possible factor in oesophageal disease progression (Macfarlane *et al.*, 2007; this study), and these organisms may also be inhibited by bifidobacteria. Research by Gibson and Wang

(1994) used plate co-culture to measure the inhibition zones of extracted bifidobacterial antimicrobial substances with a range of enteropathogens, including campylobacters. *Bifidobacterium angulatum*, *B. infantis* and *B. longum* exerted the greatest inhibitory effects on campylobacter growth, again indicating that these probiotic bacteria could be used instead of, or in conjunction with, antibiotics to treat intestinal diseases. The anti-inflammatory nature of bifidobacteria has also been studied with a view for their possible use in treatment of ulcerative colitis (Furrie *et al.*, 2005a). This study provided patients with either a synbiotic (*B. longum* and Synergy 1) or a placebo for one month, taking biopsies before and after treatment to measure modifications in the host response. This synbiotic therapy upregulated β-defensins, and reduced the expression of TNF-α and Il-1α in the treatment group (Furrie *et al.*, 2005a).

Research by Collado *et al.* (2005) found that antimicrobial peptides produced by bifidobacteria are also active against *H. pylori*. Six bifidobacterial strains were isolated from human faeces, and their cell-free supernatants were tested in co-culture with *H. pylori* from gastric biopsies, to measure the resultant inhibition zones. These strains produced inhibitory peptides, such as bacteriocins, however, they were not further classified by this research. Additionally, these bacteria were resistant to the majority of antibiotics, and could potentially be used in the treatment of *H. pylori* associated gastritis. These studies suggest a possible role for probiotic therapy in oesophageal disease; however, consideration of the effects of bifidobacteria on *H. pylori* must be taken into account, due to the proposed protective role of this species.

The nitrate-reducing veillonellas were diminished ten-fold in diseased patients compared with controls, while fusobacteria were reduced 100-fold between control and GORD patient cohorts. This pattern of reduction in GORD, before an increase back to normal numbers and spread in BO and cancer is similar in many of the bacterial groups studied (including staphylococci, lactobacilli and bifidobacteria). However, for veillonella and fusobacteria, numbers were lower in cancer patients than in controls, similar to *H. pylori*. Veillonella and fusobacteria can co-aggregate, with veillonellas being a secondary coloniser and fusobacteria, a bridge organism in biofilm development. *Veillonella* only weakly adhere to epithelial surfaces, relying on other species to which they can co-aggregate with: mainly streptococci and actinomyces. A recent study by Periasamy and Kolenbrander (2010), investigated single and multi-species colonisation, finding an increased presence of multi-species biofilms when veillonella were present, indicating a promotion of mutualistic community development by this species. Additionally, studies of oral populations show that this genus only adhere to species present in the same niche, with veillonellas from the tongue only adhering to other bacteria on the tongue, not from other surfaces (Kolenbrander, 2006). This spatio-temporal preference for inter-bacterial adherence may also be important for oesophageal communities. Oral bacteria from all sites are washed into the oesophagus and therefore, distinct co-aggregates and biofilms may only develop between certain species, dependent on the primary colonisers of the oesophageal mucosa and their original habitat. This mechanism may be important in understanding the unique microbiota that develops in the oesophagus, and its variability between each individual.

This loss in prevalence of veillonella, fusobacteria and *H. pylori* in diseased patients, particularly in the cancer cohort, indicates a change in whole biofilm structure. Further research into the prevalence of streptococci and actinomyces in these subjects is required to better understand the ecological significance of these early colonisers, and the detrimental effect to the microbiota due to their displacement. In the molecular study by Yang *et al.* (2009), streptococci constituted the majority of the 'Type I' microbiota in normal patients, with a reduction in this genus and other Gram positive species, with disease progression ('Type II'). Loss of this genus could initiate a further change in biofilm structure, with bridge organisms, such as fusobacteria, being equally reduced.

Similar to data from Chapter 2, *Campylobacter* prevalence increased in diseased subjects, with a 10-fold difference in means between GORD and cancer patients. Only three cancer patient biopsies yielded results for the presence of campylobacter. However, this was most likely due to issues with the assay, with results from Chapter 2 indicating an increase in prevalence of this species with disease progression. Lack of data for cancer patients, and their presence in cancerous and non-cancerous matched tissues is unfortunate, and further research is required with these patients.

Studies into the cytokine responses of oesophageal tissues in health and disease produced a large variation in results for each patient, with no significant differences found. This is in contrast to previous studies into the host responses (Fitzgerald *et al.*, 2002a, 2002b; Isomoto *et al*., 2003; O'Riordan *et al.*, 2005), where IL-1β and IL-8 were increased in disease, or shifts in their expression were correlated (Section 3.1.3). In the

present study few results were obtained for normal squamous tissue in cancer patients, and no relationships were identified between GORD grades or presence of *Campylobacter* species. Investigation of the host response in oesophageal disease by Fitzgerald *et al.* (2002b) used PCR with densitometry analysis of the resulting bands to quantify levels of total RNA for each cytokine. The primers used for PCR (Jung *et al.*, 1995) were also tested for use in this study, however, unspecific bands were identified in some samples and low levels of primer-dimer were expressed. Fitzgerald *et al.* (2002b) also employed ELISA, finding a correlation between relative differences in cytokine expression, although data from immunostaining was not formally quantified. The study by O'Riordan *et al.* (2005) also used ELISA to measure cytokine levels. This technique measures protein rather than gene expression, with no direct correlation between mRNA expression and protein production (Mehra *et al.*, 2003). The concentration of a protein is determined not only by mRNA but also by its related translation to protein, and additional factors involved in protein degradation. This, together with primer issues, could explain the differences in results between these studies and the present research. Therefore, to fully assess the cytokine response in oesophageal health and disease, analysis of both gene expression and protein level is required.

TNF-α is expressed in specific areas of tissues and glands, and therefore, only in certain cells of metaplastic or dysplastic regions (Tselepis *et al.*, 2002). Consequently, in a whole biopsy, cell-cell variation in expression may occur, and measurement of expression in the biopsy could mask increased expression in specific cells. In addition, the inflammatory gradient between proximal and distal sites of the diseased tissue may

account for these data (Fitzgerald *et al.*, 2002a). Individual host responses, genetics and variations in the microbiota may be important factors in the inflammatory response to oesophageal disease. With such a diverse range of results for each cytokine in this study, further genetic and proteomic analysis is required to fully investigate these interactions.

As with Chapter 2, no definite conclusions can be made from this microbiological or immunological data, with no aetiologic agent identified. However, these results correspond with previous research of the oesophageal microbiota, indicating a shift with disease progression, and a conformational change in biofilm composition, represented by changes in prevalence of certain species and their co-aggregation partners. The ability of *H. pylori* to induce apoptosis in ADC cells, and the reduction of this species in oesophageal disease is of great interest, indicating that the progression of disease to cancer could depend on the host's microbiota, rather than changes in the epithelia leading to a shift in the microbiome.

The question remains; does the host's individual microbiota initiate disease progression when exposed to reflux, or, do the changes in epithelial cell type result in the modification of bacterial populations? A long-term study of individuals at risk of oesophageal disease, and the alteration in each subject's microbiota during progression, could be the key to understanding the involvement of bacteria in oesophageal dysfunction.

Chapter 4

Chemostat model of the oesophagus to investigate the effects of refluxate on microbiota composition and its associated pathogenicity

4.1 Introduction

4.1.1 In vitro model systems

Chemostats have been used in research and industry for over fifty years, for investigation of a single bacterium to a whole gut microflora, and planktonic to biofilm populations. However, use of these *in vitro* systems declined in the last thirty years when genetic techniques became increasingly popular. Modern genetics has allowed much greater understanding of microbial genomes, protein, mRNA and metabolite profiles, providing a molecular understanding of microbial physiology. These techniques have employed batch culture to obtain bacterial cells, however, recent studies have shown that this growth method can alter cell physiology and gene expression (ter Linde *et al*., 1999; Causton *et al*., 2001). Therefore, the use of chemostat cultures in conjunction with genomic and proteomic approaches provides a more accurate representation of microbial processes in their natural environment (Wick *et al*., 2001; Piper *et al*., 2002).

4.1.2 In vitro models of the oral cavity

Since the early 19th century 'artificial mouth' models have been in development, progressing from basic and simple apparatus, to that of computerised multi-station artificial mouth systems (Tang *et al.*, 2003). In 1976, Dibdin and co-workers used continuous culture apparatus to model the oral cavity and dental plaque development (Dibdin *et al.*, 1976). Since this time, *in vitro* continuous culture systems have been used extensively to model the oral microbial community, allowing full, controlled investigation of biofilm composition and bacterial metabolism (Marsh *et al.*, 1983;

McKee *et al.*, 1985). Studies which use plaque, or two or three defined species for inoculation are not necessarily reproducible, due to the variation in plaque composition over time. Additionally, the recovery of samples can disturb biofilm communities, therefore, this latter study aimed to overcome these issues. Nine oral organisms (*Streptococcus mutans*, *S. sanguis*, *S. mitior*, *Veillonella alcalescens*, *Actinomyces viscosus*, *Bacteroides intermedius*, *Fusobacterium nucleatum*, *Neisseria* sp. A1078 and *Lactobacillus casei*) were employed to determine the effect of glucose-excess and glucose-limited environments on microbial establishment, and whether these models could generate reproducible results (McKee *et al.*, 1985). When glucose was limited (28 mmol l^{-1}) all nine species were established and maintained in the planktonic environment, with *Streptococcus mitior* and *Veillonella alcalescens* predominating. However, in an excess of glucose (390 mmol l^{-1}) species diversity was reduced, with *L. casei* being the predominant bacteria. Biofilm samples also developed on the surface of the chemostat vessel, with interactions observed between fusobacteria, veillonellas and streptococci. The growth of anaerobic fusobacteria and veillonella indicate that other species sequestered oxygen, providing conditions for survival. Additionally, the increased diversity under glucose limitation suggests that competition for nutrients is an important factor for survival, requiring a mix of substrates for development of complex communities. This study provided insight into the communications between bacteria when in juxtaposition, and the protection provided by a biofilm.

This research group continue to use chemostat models for investigation of the role of oral bacteria in health and disease, investigating the development of biofilms and the

effects of carbohydrates, nutrient availability and oxygen on bacterial growth rates (Bradshaw *et al.*, 1989, 1996a, 1996b). These studies highlighted the effect of environmental changes on species composition, and the disruption of biofilms under nutrient and pH stress. The bacteria, veillonella, *S. mutans* and *L. casei* increased significantly when exposed to pulses of carbohydrate, especially when pH was not controlled, producing an acidic environment (Bradshaw *et al.*, 1989). Additionally, anaerobe presence was increased as biofilms aged, suggesting that the complex communities offered protection to obligate anaerobes from the toxic effects of oxygen (Bradshaw *et al.*, 1996a, 1996b).

4.1.3 Bacterial responses to the environment

Bacteria colonise a large variety of niches with differing environmental conditions. Inside the host, they may encounter unfavourable conditions such as nutrient and oxygen depletion or abundance, acidity and presence of bile acids and digestive enzymes. Bacteria possess a number of strategies to over come these conditions, often producing virulence factors for survival. Under stress, organisms can produce toxins such as haemolysins, allowing acquisition of iron, and mucinolytic enzymes to further degrade oligosaccharides in mucus for energy.

Microorganisms present throughout the gastrointestinal tract must be capable of synthesising a variety of hydrolytic and mucinolytic enzymes, enabling the breakdown of surface mucus. The mucins present in epithelial mucus layers are composed of large quantities of galactose and hexosamines, with fucose, sialic acids (neuraminic acid) and

sulphate groups present in variable proportions (Macfarlane *et al.*, 2005). The presence of sulphate and sialic acids confers resistance to glycosidase degradation; therefore, bacteria must also produce proteases, peptidases and sulphatases additional to enzymes for breakdown of sugar complexes.

A study of the stress response in *Clostridium septicum*, a highly pathogenic species, identified a catabolite regulated mucinolytic response to nutrient depletion. During stress, these bacteria transformed to swarm cells, with increased virulence and toxin production (Macfarlane *et al.*, 2001), producing mucinolytic enzymes and haemolysins. A more recent study with *C. jejuni*, found that when exposed to MUC2, the most common mucin secreted by goblet cells in the gastrointestinal tract, there was an up-regulation of genes for colonisation and pathogenicity (Tu *et al.*, 2008). Genes for cytolethal distending toxins, cytotoxin, motility and mucin degradation were all upregulated, however, with further increases in concentration of this mucin, total bacterial CFU was reduced. This could be due to the bacteriocidal activities of certain mucin oligosaccharides, as evidenced by the inhibition of *H. pylori* growth by α1,4-linked *N*-acetylglucosamine in the gastric mucin, MUC6 (Kawakubo *et al.*, 2004).

Through degradation of carbohydrates in the epithelial mucus layer, the function of mucin is lost, allowing invasion and attachment to epithelial cells. The surface structures of mucins are variable throughout the GI tract both intra- and inter-individually, both in humans and animals. These differences may explain the variation in response of bacteria throughout diverse niches and environmental conditions.

Chemostats have been used to model oral, gastric (O'May *et al.*, 2005b) and colonic (Macfarlane *et al.*, 1998) communities; however, this study represents the first chemostat for study of the oesophageal microbiota. This model was designed using the hypothesis that the major bacteria colonising the oesophagus originate in the oral cavity, with selected species adhering to the mucus layer of this organ, rather than washing into the stomach. Therefore, a two-stage system was developed, containing a vessel (oral, A), held above a second (oesophagus, B), allowing gravitational flow, representative of the human structure. Inside each vessel, discs were suspended to allow biofilm formation: hydroxyapatite (mineral in tooth enamel) and artificial mucin gels (representing mucus layer), respectively. Following five days of biofilm development, these mucin gels, representative of the colonising oesophageal microbiota, were exposed daily to a bile acid solution.

This research firstly aimed to design an *in vitro* model representative of the normal oesophageal microbiota, and secondly, to investigate the effect of refluxate on these communities. It was hypothesised that, if an oral community was generated in vessel A, consequently, under the nutrient deplete conditions of vessel B, a varied population of bacteria, similar to that in the oesophagus would establish. Furthermore, due to the exposure of these biofilms to acid and bile, in similar time periods to those seen clinically, the bacterial community would be modified, selecting for acid and bile tolerant species. Additionally, this microbiota would be more representative of that seen in disease patients, becoming progressively Gram negative, with increased production of virulence proteins, and enzymes for substrate acquisition.

4.2 Materials and Methods

4.2.1 In vitro modelling system

A chemostat two-stage model of the oesophagus was designed to attempt to establish a biofilm representative of the oesophagus through colonisation with oral bacteria. Two glass vessels each with a working volume of 480 ml were connected in series (Fig. 4.1). Media contained in g L^{-1}: type III mucin 0.25, casein 0.25, bacteriological peptone 1.0, yeast extract 2.5, glucose 1.0, tryptone soy broth 0.1, NaCl 4.5, KCl 1.5, KH_2PO_4 1.0, $MgCl_2$ 0.125, $CaCl_2$ 0.15, hemin 0.005, cysteine 0.5, vitamin K1 0.01. Media was autoclaved and cooled on a stirrer before addition of a filter-sterilised solution containing potassium nitrate, sodium succinate, di-sodium fumarate and sodium formate, all 1g L^{-1}.

Both oral (A) and oesophageal (B) vessels (Fig. 4.1) were sterilised for 20 min containing the above media, with all equipment attached. Each vessel possessed a pH meter, two inlets for acid/alkali addition, a gas inlet for attachment of nitrogen, and a gas outlet filter to allow removal of excess gas produced. Once both vessels were cooled, the oral vessel (A) was connected to the oesophagus (B), and the latter attached to a sterile waste vessel. Culture pH was controlled at 7.0 by addition of 1M HCl and 2M NaOH using a pH 1000 pH system (New Brunswick Scientific, St. Albans, Herts, UK), attached to a CW711/EXT/250 pH electrode (Thermo-Russell, Auchterarder, UK). Vessels were sparged with O_2-free N_2 gas to keep a headspace (10 cm^3 min^{-1}), and a 37^oC water-jacket maintained temperature in each vessel (Haake B3 recirculating water bath, Fischer Scientific UK Ltd., Loughborough, UK).

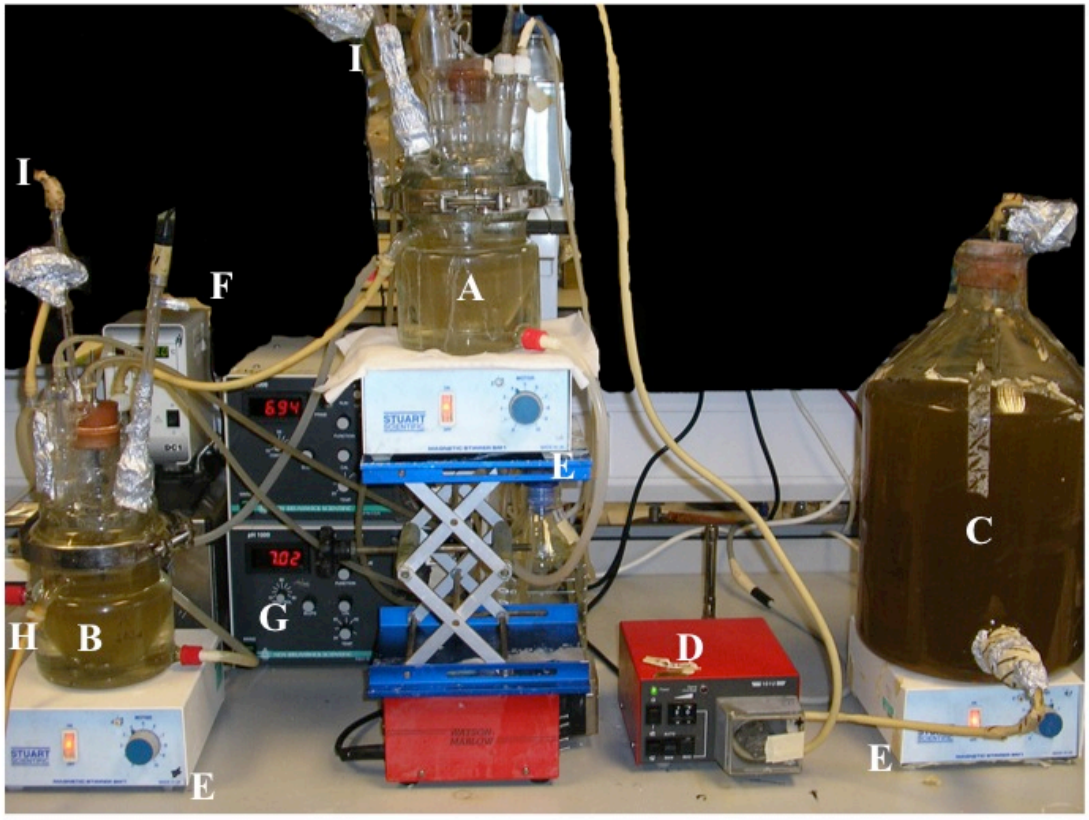


Fig. 4.1: *Image of model oral-oesophageal chemostat system. Oral chemostat (A), connected above oesophageal chemostat (B), with fresh media (C) pumping in to the oral chemostat via pump (D). All media and chemostat vessels were continuously stirred (E), and temperature and pH controlled through a water bath (F) and two pH pumps (G). Waste removed from the oesophageal chemostat via tubing (H). Nitrogen gas was introduced to each vessel through a flow cell to keep a headspace of 10 cm^3 min^{-1} (I).*

4.2.2 Establishment of an oesophageal biofilm

Fresh saliva from a healthy volunteer (female, 26 years old, with no known signs or symptoms of periodontal disease) was used to inoculate the oral vessel. 10 - 15 ml was collected and added to the oral vessel and left overnight to establish. Twenty-four hours later oral vessel A was allowed to flow over into the oesophageal vessel (tubing unclamped). Fresh medium (C) was pumped into vessel A at a dilution rate of 0.1 h^{-1}. This subsequently fed vessel B, which was consequently carbohydrate depleted.

Cultures were grown for 5 days (12 turnovers) to allow achievement of a steady state before samples were taken from the planktonic phase for viable counts of bacteria on a selection of agar plates. At the time of this sample sterile porcine mucin gels (2% mucin type III, 0.8% bacteriological agar) in metal discs (0.8 cm diameter) were placed in the oesophageal vessel on metal plates suspended on the central rubber bung. To investigate the biofilm potential in the oral chemostat hydroxyapatite discs (Clarkson Chromatography, PA, USA) were placed inside the metal discs, held with 1% bacteriological agar.

Planktonic phase samples were vortexed to homogenise any bacterial aggregates and serially diluted in half strength peptone water. Dilutions of neat to 10^{-8} were plated onto a variety of selective and non-selective agars: Nutrient agar, Blood azide (both aerobic), Chocolate agar (microaerophilic growth), Columbia blood agar supplemented with succinate, nitrate, formate and fumarate (1 g L^{-1} each), Anaerobe basal blood agar (microaerophilic and anaerobic growth), Wilkins-Chalgren blood agar (plain and Gram negative supplements), Beerens (Beerens, 1990), Rogosa and Reinforced clostridial agar (all anaerobic). All blood plates contained 5% (v/v) defibrinated horse blood, with all agars, supplements and blood purchased from Oxoid, Basingstoke, UK.

Mucin and hydroxyapatite discs were left for 48 hours to allow attachment and invasion of bacteria and establishment of a biofilm. At 48 hours sample discs were removed from each vessel and biofilms broken down with a glass homogeniser and 9 ml half strength peptone water before plating out as above.

Hydroxyapatite discs were removed from the oral vessel on days 7, 13 and 21 (2, 8 and 16 days of biofilm growth respectively), to monitor the development of the biofilm over time. Oesophageal biofilm samples were taken on days 7, 11 and 12 (2, 5 and 6 days of growth respectively) in the initial experiment. When repeated, oral biofilms were measured on days 7 and 16 (2 and 11 days growth), while mucin biofilms were removed on days 7, 11, 12, 15 and 20. Exposure of mucin traps to bile acid solution commenced on day 11 of both experiments, and these model oesophageal biofilms were sampled on day 1 at 0 and 8 hours, day 2 after overnight recovery (test 1) and days 4 and 9 of exposure (test 2) (Table 4.1).

4.2.3 *Exposure to acid and bile salts*

Bile salts No. 3 (Oxoid, UK) (0.4 g L^{-1}) was made to pH 3 (1 M HCl) and filter sterilised. After mucin traps had established a biofilm for 5 days they were removed and exposed to a petri dish of this solution for 30 min at 2 hour intervals, over an 8 hour time period. This was carried out each day for a planned 14 days, with samples taken throughout for microbiological, haemolytic and enzymatic analysis. Additionally, after 6 days of exposure to this bile acid cocktail, control and exposed biofilms were removed for tests with cell culture (Chapter 5). Table 4.1 describes all steps taken throughout each run of experimentation.

4.2.4 Characterisation of bacterial isolates

The MIDI system was employed for identification of all isolated bacteria, as described in Section 2.2.3 of Chapter 2.

4.2.5 Haemolysis assay

A mucin trap was removed from vessel B and homogenised with 10 ml half strength peptone water, and utilised for total bacterial counts, haemolysis, glycosidase and neuraminidase assays. A solution of 24 ml NaCl and 0.5 ml $CaCl_2$ was added to 0.5 ml defibrinated horse blood (Oxoid, UK), 0.9 ml of this was added to 0.1 ml of sample and incubated at 37^{o}C for 1 hour. This was centrifuged at 15,000 *g* for 30 s before measuring absorbance at 540 nm (Cecil BioQuest CE 2501, Bath, UK) (Allison *et al.*, 1992). One unit of haemolytic activity resulted in an increase in absorbance of 0.1 h^{-1}. Results are expressed as units per $1x10^8$ CFU (U $\log_{10}$ 8 CFU^{-1}).

4.2.6 Glycosidase assay

N-acetyl-β-D-glucosaminidase, β-galactosidase and α-fucosidase levels were measured by monitoring the release of chromogen from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-α-D-fucopyranoside respectively (15 mM in 0.01 M Tris buffer at pH 6.5). For each assay, 0.5 ml of the respective *p*-nitrophenyl substrate (above) was mixed with 0.25 ml of homogenated biofilm sample (Section 4.2.5); these were incubated at 37^{o}C for 1 hour. A solution of 0.5 M Na_2CO_3 and 0.5 M $NaHCO_3$ was added to stop the reaction (0.75 ml) and the absorbance was read at 420 nm. Tris buffer was used as a blank, with controls of the substrate and buffer alone, and sample and buffer run simultaneously. A standard curve was used to calculate enzyme activities using *p*-nitrophenyl (4-nitrophenol) dilutions from 1-1000 nM in Tris buffer. One unit of glycosidase activity corresponded

to 1 μmol of *p*-nitrophenyl released h^{-1}. Results are expressed as units per $1x10^8$ CFU (U $\log_{10}$ 8 CFU^{-1}).

4.2.7 Neuraminidase assay

Neuraminidase (sialic acid) production was measured using the substrate *N*-acetyl neuraminlactose (1 mg ml^{-1} in 0.1 M acetate buffer at pH 5.5). 0.1 ml of this solution was added to 0.05 ml of homogenised biofilm (Section 4.2.5), with two controls of 0.05 ml boiled sample with substrate and 0.05 ml acetate buffer with substrate. These were incubated at 37°C for 1 hour before boiling for 2 min to stop the reaction. The solution was developed in three stages: 0.05 ml of solution A (0.2 M sodium-meta-periodate in 9 M phosphoric acid) was mixed and left to stand for 20 min at room temperature, 0.5 ml of solution B (10% sodium arsenite in solution of 0.5 M sodium sulphate, 0.1 N sulphuric acid) was added and shaken to remove the resulting yellow/brown colour, 1.0 ml of solution C (0.6% thiobarbituric acid in 0.5 M sodium sulphate) was added and shaken before boiling for 15 min. After development, tubes were centrifuged at 13,000 *g* for 3 min and the absorbance read at 549 nm. A standard curve was constructed using 1 mg ml^{-1} *N*-acetylneuraminic acid in 0.1 M acetate buffer (pH 5.5). Dilutions were made from 0 - 1 mg ml^{-1} and developed as above. One unit of neuraminidase activity was defined as 1 μg of *N*-acetylneuraminic acid released h^{-1}. Results are expressed as units per $1x10^8$ CFU (U $\log_{10}$ 8 CFU^{-1}).

4.2.8 FAME analysis

At intervals throughout experimentation (Table 4.1), 4 ml of planktonic phase medium was removed from each vessel, the samples were centrifuged at 2600 rpm for 15 min, supernatant was frozen at -20^{o}C for future analysis, and the pellet was left in an oven for 2 days to determine dry weight. Due to time constraints analysis could not be carried out on these samples, however, may be used in future to obtain information on production of SCFA and whether a steady-state was obtained in this chemostat model.

4.2.9 Statistical analysis

Statistical analysis was conducted using Prism Statistical Package (Section 2.2.5). Data were analysed using unpaired Student's t-tests, with a normal distribution assumed (K-S test). A P-value of <0.05 was classed as significant.

Table 4.1: *Chemostat model of the oral cavity and oesophagus – timetable of experimentation.*

Day	Procedures for Test 1	Procedures for Test 2
0	Fresh saliva added to oral vessel	Fresh saliva added to oral vessel
1	Oral vessel allowed to flow to oesophageal vessel and media connected	Oral vessel allowed to flow to oesophageal vessel and media connected
2 -4	Left to establish	Left to establish
5	Planktonic samples taken and mucin/hydroxyapatite discs added	Planktonic samples taken and mucin/hydroxyapatite discs added
6	Left to establish	Left to establish
7	Biofilm samples taken (2 days growth)	Biofilm samples taken (2 days growth)
8-10	Left to establish	Left to establish
11 (Day 0 of exposure)	Oesophageal biofilm samples taken at 0 and 8 h of exposure for bacterial counts	Oesophageal biofilm samples taken at 0 and 8 h of exposure for bacterial counts, haemolysis and enzyme assays[a]
12 (1)	Bile/acid exposure Oesophageal biofilm sample taken after overnight recovery	Bile/acid exposure Planktonic sample for FAME analysis Oesophageal biofilm sample (recovery)
13 (2)	Bile/acid exposure Oral biofilm sample taken (8 days growth)	Bile/acid exposure
14 (3)	Bile/acid exposure	Bile/acid exposure
15 (4)	Bile/acid exposure Planktonic samples taken for FAME analysis	Bile/acid exposure Bacterial counts, enzyme and haemolysis assays, planktonic samples taken for FAME analysis
16 (5)	Bile/acid exposure	Bile/acid exposure Oral biofilm sample (11 days growth)
17 (6)	Bile/acid exposure Cell line tests with OE21 and FLO-1 cells (exposed biofilm)	Bile/acid exposure Cell line tests with OE21, FLO-1 and CP-A cells (exposed and control biofilm)
18 (7)	Bile/acid exposure Oesophageal sample taken	Bile/acid exposure
19 (8)	Bile/acid exposure	Bile/acid exposure
20 (9)	Bile/acid exposure	Bile/acid exposure Bacterial counts, enzyme and haemolysis assays, planktonic samples taken for FAME analysis
21 (10)	Bile/acid exposure Oral biofilm sample taken (16 days growth	Bile/acid exposure pH electrode malfunction
22 (11)	Bile/acid exposure Water bath failed to re-circulate water	

[a] *Enzyme assays – both glycosidase and neuraminidase assay tests.*

4.3 Results

4.3.1 Mechanical failures during experimentation

An *in vitro* continuous culture system was designed to model oral and oesophageal bacterial communities using fresh saliva. Microbial identification with FAME profiles was utilised to characterise the bacteria isolated in these continuous culture systems. During the first experimental run, the water bath pump malfunctioned, it is possible this occurred prior to day 22, and therefore experimental data for day 21 is somewhat questionable. The experiment was repeated under identical conditions with temperature and pH being monitored daily to identify any further mechanical failure. The pH electrode malfunctioned on day 21 of the second run (day 10 of refluxate testing), resulting in the oesophageal vessel being washed out with 1 M HCl. Therefore, the experiment was stopped at this time and could not be repeated again due to time constraints. The gas chromatography machine failed to calibrate at the start of bacterial characterisation for organisms isolated in the second experiment. Despite efforts, this machine could not be used for determination of these bacterial isolates; therefore, no microbiological data is available for the second chemostat experiment.

4.3.2 Establishment of a model oral and oesophageal microbiota

Figure 4.2 shows the total viable counts obtained in both sets of experiments for the initial planktonic and biofilm populations, with similar mean $\log_{10}$ CFU established in both. Statistically significant variations were identified between bacterial populations in these vessels: oral planktonic and biofilm ($P < 0.0001$), oesophageal planktonic and biofilm ($P = 0.0028$), and oral and oesophageal planktonic phase ($P = 0.0015$).

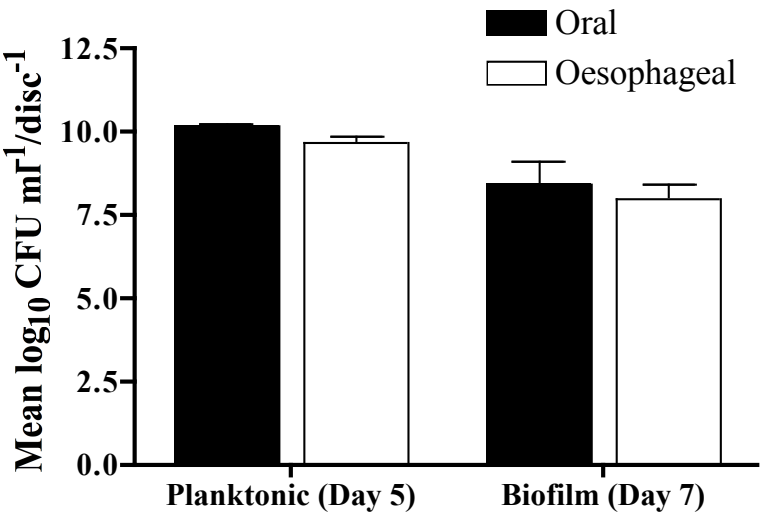


Fig. 4.2: *Graph represents mean log_{10} CFU ml^{-1} (planktonic) and mean log_{10} CFU $disc^{-1}$ (biofilm on hydroxyapatite and mucin gels, both 0.8cm diameter). Error bars represent 4 sets of data from two experimental runs with duplicate total counts from WC blood plates.*

Microbiological data from the first experimental run shows that within five days a complex population of species was established in this chemostat model. *Corynebacterium matruchotii* and *Veillonella parvula* comprised the greatest mean log_{10} CFU ml^{-1} of culturable bacteria in the planktonic phase of the oral and oesophageal vessels respectively (Table 4.2). Streptococci had the greatest species variation with seven different species isolated throughout experimentation (Tables 4.3 and 4.4), while *S. gordonii* was isolated from only the oral vessel. In the planktonic phase *Gemella* and *Eubacterium* were found only in the oral vessel, while *Staphylococcus* and *Veillonella* singularly colonised the oesophageal system. Streptococci, corynebacteria and fusobacteria were isolated from both vessels of this system, with higher numbers of all three genera found in the oral planktonic phase (Table 4.2). Mucin and hydroxyapatite platforms were placed in the oesophageal and oral vessels respectively, on day 5, with samples removed over time to measure biofilm development. After two days of biofilm

establishment, oral and oesophageal proportions of total streptococci colonised these surfaces similarly, at numbers ca. $\log_{10}$ lower than in the planktonic phase. *Corynebacterium*, *Gemella* and *Eubacterium* species were not identified in these biofilm populations, while staphylococci and veillonella were isolated from the biofilm of both models. *Faecalibacterium prausnitzii* and *Rothia denticariosa* were also isolated, with the latter only in mucin gels (Tables 4.2-4.4).

Table 4.2: *Composition of planktonic and biofilm populations in chemostat vessels modelling the oral and oesophageal microbiota* [a].

Genus	Planktonic (Day 5)		Biofilm (Day 7)	
	Oral	Oesophageal	Oral	Oesophageal
Streptococcus	9.4 ± 0.3	8.4 ± 2.1	8.3 ± 0.4	6.9 ± 0.5
Corynebacterium	9.7 ± 0.0	8.8 ± 0.0	-	-
Gemella	8.2 ± 0.0	-	-	-
Fusobacterium	9.5 ± 0.5	8.6 ± 0.1	8.2 ± 0.3	6.6 ± 0.5
Eubacterium	8.2 ± 0.0	-	-	-
Staphylococcus	-	3.4 ± 0.0	8.4 ± 0.0	6.0 ± 0.0
Veillonella	-	9.2 ± 0.0	8.6 ± 0.0	6.0 ± 0.0
Faecalibacterium	-	-	7.6 ± 0.0	7.2 ± 0.0
Rothia	-	-	-	6.0 ± 0.0

[a] *Data represents mean $\log_{10}$ CFU ml^{-1} ± SD (planktonic) and mean $\log_{10}$ CFU $disc^{-1}$ ± SD (biofilm on mucin gels, 0.8 cm diameter).*

4.3.3 Effect of bile acid exposure on the oesophageal biofilm

A different biofilm microbiota was established in the oesophageal chemostat compared with the oral vessel. Slightly lower mean $\log_{10}$ CFU established in both vessels compared with the planktonic phase, and although similar genera were identified, there was variation in species composition. Figure 4.3 shows total counts for the oesophageal vessel over time. This data collectively represents the total counts from WC blood plates stored anaerobically during both experimental runs from duplicate culture plates. On day

2 of biofilm colonisation, numbers were $\log_{10}$ 2 CFU $disc^{-1}$ lower than in the planktonic phase (P = 0.0028), this total count remained constant for the following 4 days (day 11), however, the composition of this microbiota changed. On day 11, the diversity of streptococcal species was reduced coinciding with the loss of veillonella, rothia and faecalibacteria (Table 4.4). Following 8 hours of bile acid exposure total counts were diminished ($\log_{10}$ 7.5, P = 0.0076), however, recovered overnight ($\log_{10}$ 8.2, P = 0.0131). Over the following 8 days of exposure to bile acid solution total counts steadily reduced ($\log_{10}$ 7.0 CFU $disc^{-1}$), while species composition changed, becoming more varied and Gram negative (Fig. 4.3, Table 4.4). Statistical analyses of these total counts identify highly significant variations during the experimental model of reflux disease. Numbers after overnight recovery compared with at 0 hour of testing gave a P value of 0.0252, while recovery (day 12) vs. day four (day 15) produced a P value of 0.0312 (Fig. 4.3). From start of testing at 0 hour to days 4 and 9 of exposure (days 15 and 20, respectively), total counts were reduced highly significantly (P = 0.0009 and 0.0001).

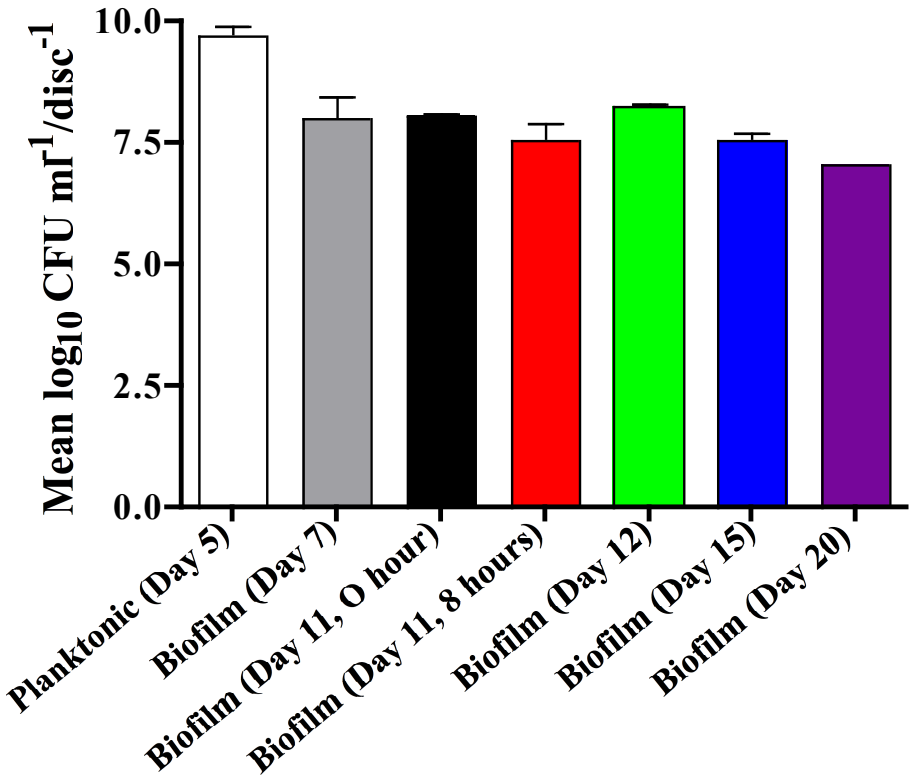


Fig. 4.3: *Graph represents mean log_{10} CFU ml^{-1} (planktonic) and mean log_{10} CFU $disc^{-1}$ (biofilm on mucin gels, 0.8 cm diameter) from the oesophageal vessel. Error bars represent four sets of data from two experimental runs with duplicate total counts from WC blood plates (only 1 set of data for days 15 and 20, test 2).*

Table 4.3: *Viable counts for bacteria isolated from the chemostat vessel representing the oral cavity (vessel A)* [a].

Bacteria	Oral planktonic (Day 5)	Oral biofilm (Day 7) [b]	Oral biofilm (Day 13) [c]	Oral biofilm (Day 21) [d]
Streptococcus				
S. mitis	8.7 ± 0.1	8.4 ± 0.1	7.0 ± 0.2	-
S. sanguinis	9.4 ± 0.1	-	7.2 ± 0.0	-
S. parasanguis	-	-	8.0 ± 0.0	-
S. intermedius	9.5 ± 0.1	8.2 ± 0.1	8.1 ± 0.0	8.4 ± 0.1
S. gordonii	9.4 ± 0.1	8.5 ± 0.1	-	-
S. oralis	9.1 ± 0.1	8.5 ± 0.0	-	-
S. anginosus	9.5 ± 0.1	8.4 ± 0.1	-	-
Staphylococcus				
Staph. warneri	-	8.4 ± 0.1	-	6.6 ± 0.0
Staph. epidermidis	-	-	4.0 ± 0.0	8.5 ± 0.0
Fusobacterium				
F. nucleatum	9.7 ± 0.0	-	-	-
F. necrophorum ss necrophorum	9.0 ± 0.1	8.3 ± 0.4	9.3 ± 0.0	8.8 ± 0.3
F. nariforme	-	8.0 ± 0.0	-	-
Corynebacterium				
C. matruchotii	9.7 ± 0.0	-	-	7.8 ± 0.2
Faecalibacterium				
F. prauznitzii	-	9.6 ± 0.0	-	8.6 ± 0.3
Gemella				
G. haemolysans	8.2 ± 0.2	-	-	-
Eubacterium				
E. S17	8.2 ± 0.2	-	-	-
Veillonella				
V. parvula	-	8.6 ± 0.0	-	-
Peptostreptococcus				
P. micros	-	-	-	8.0 ± 0.1
Bifidobacterium				
B. breve	-	-	7.4 ± 0.2	-
B. D05	-	-	-	7.4 ± 0.1
B. D02A	-	-	-	7.0 ± 0.0
Actinomyces				
A. odontolyticus	-	-	8.2 ± 0.1	-
A. bovis	-	-	-	8.2 ± 0.1
Gardnerella				
G. vaginalis	-	-	-	8.0 ± 0.0
Clostridium				
C. clostridiforme	-	-	-	8.0 ± 0.0

[a] Numbers represent the mean log_{10} CFU ml^{-1} ± SD (planktonic) and mean log_{10} CFU $disc^{-1}$ ± SD (0.8 cm diameter hydroxyapatite discs for bacterial attachment). [b] Day 7 of chemostat - 2 days biofilm growth on hydroxyapatite discs. [c] Day 13 of chemostat – 8 days biofilm growth on hydroxyapatite discs. [d] Day 21 of chemostat – 16 days biofilm growth on hydroxyapatite discs.

Table 4.4: *Viable counts for bacteria isolated from the chemostat vessel representing the oesophagus (vessel B)* [a].

	Oesophageal planktonic (Day 5)	**Oesophageal biofilm (Day 7)** [b]	**Oesophageal biofilm (Day 11)** [c]	**Oesophageal biofilm (Day 11)** [d]	**Oesophageal biofilm (Day 12)** [e]
Streptococcus					
S. mitis	8.2 ± 0.2	6.4 ± 0.1	-	-	6.3 ± 0.0
S. sanguinis	5.2 ± 0.0	7.2 ± 0.2	-	-	-
S. parasanguis	-	-	6.0 ± 0.1	6.7 ± 0.1	5.4 ± 0.1
S. intermedius	9.6 ± 0.0	7.3 ± 0.0	6.7 ± 0.1	6.9 ± 0.1	7.2 ± 0.1
S. oralis	9.6 ± 0.1	7.2 ± 0.1	-	-	6.3 ± 0.0
S. anginosus	-	6.3 ± 0.0	-	5.5 ± 0.3	-
Staphylococcus					
Staph. warneri	3.4 ± 0.2	6.0 ± 0.0	6.1 ± 0.2	6.1 ± 0.1	6.5 ± 0.2
Staph. epidermidis	-	-	6.9 ± 0.0	-	6.4 ± 0.0
Fusobacterium					
F. nucleatum	8.5 ± 0.0	-	7.2 ± 0.1	-	-
F. nucleatum ss vincentii	-	6.6 ± 0.0	6.0 ± 0.0	-	6.6 ± 0.6
F. necrophorum ss necrophorum	8.6 ± 0.4	7.0 ± 0.0	6.4 ± 0.2	-	7.8 ± 0.0
F. russii	-	6.0 ± 0.0	-	-	-
Corynebacterium					
C. matruchotii	8.8 ± 0.2	-	-	5.0 ± 0.0	-
Faecalibacterium					
F. prauznitzii	-	7.2 ± 0.2	-	-	-
Veillonella					
V. parvula	9.2 ± 0.0	-	-	-	-
V. atypica	-	6.0 ± 0.0	-	-	
Rothia					
R. denticariosa	-	6.0 ± 0.0	-	-	-
Peptostreptococcus					
P. micros	-	-	6.3 ± 0.0	-	-
Actinomyces					
A. D01	-	-	-	5.0 ± 0.0	7.4 ± 0.0
Prevotella					
P. tannerae	-	-	5.5 ± 0.2	6.4 ± 0.1	-
Propionibacterium					
P. acnes	-	-	-	-	6.9 ± 0.1
Clostridium					
C. malenominatum	-	-	6.0 ± 0.1	-	-
Capnocytophaga					
C. sputigena	-	-	5.8 ± 0.2	5.8 ± 0.1	6.9 ± 0.0
Campylobacter					
C. concisus	-	-	-	6.4 ± 0.1	-
Selenomonas					
S. infelix	-	-	-	-	6.0 ± 0.0

[a] Numbers represent the mean log_{10} CFU ml^{-1} ± SD (planktonic) and mean log_{10} CFU $disc^{-1}$ ± SD (0.8 cm diameter mucin gels for biofilm establishment). [b] Day 7 of chemostat - 2 days biofilm growth on mucin gels. [c] Day 11 of chemostat – 6 days biofilm growth on mucin gels, 0 hour before bile acid exposure. [d] Day 11 of chemostat – following 8 hours of bile acid exposure. [e] Day 12 of chemostat – 7 days biofilm growth, overnight recovery from bile acid exposure.

Despite a lack of microbiological data for the second experimental run, Fig. 4.2 highlights the similarity in total counts between these two tests. Morphological observation of the species isolated in the oral and oesophageal vessels of the second run were similar to those of the first, indicating that these two tests produced analogous results. On day 2 of biofilm formation similar total counts were found in all vessels. However, in the second run tetracocci were isolated on Anaerobe basal blood agar, and branching rods, possibly bifidobacteria or actinomyces were isolated from the oral vessel. Following 8 hours of periodic washings of the oesophageal biofilm with bile acid cocktail, thin straight Gram negative rods were observed, which were suspected to be fusobacteria or leptotrichia. Tiny, thin and curly Gram negative rods were isolated from this sample, suggesting that campylobacter and capnocytophaga were selected due to refluxate, comparable to the first test (Table 4.4).

After four days of exposure to bile acids for 8 hours a day, morphological observations suggest that the presence of Gram positive organisms was significantly reduced, with very little coccal species found. Studies of bacterial isolates indicates that they were replaced by Gram negative species, with both rod and tiny cocci morphologies. Furthermore, on day 20 (day 9 of exposure) tetracocci, tiny cocci and a great range of rod morphologies were observed. Utilising information from the first run and data from Chapter 2 (Table 2.2 – 2.4), the genera *Micrococcus*, *Veillonella*, *Neisseria*, *Fusobacterium*, *Leptotrichia*, *Campylobacter*, *Capnocytophaga* and *Selenomonas* were proposed.

4.3.4 Global populations

Figures 4.4 and 4.5 show the percentage that each bacterial group constituted during the stages of community development, for the oral and oesophageal models respectively, in the initial experimental run. These data provide a global view of the microbial composition of each sample.

Streptococci represent the greatest proportion of the planktonic phase in both the oral and oesophageal vessels (Figs. 4.4 and 4.5), comprising 55%, and 45%, respectively. By day 21, streptococci had reduced to 9% of the oral biofilm population from ca. 50% on days 7 and 13, although this may represent the detrimental effect of the water bath malfunction. Fusobacteria comprised 19% of the planktonic phase, with equal representation in the oral biofilms of days 7 and 13, reducing slightly to 9% by day 21. *Corynebacterium matruchotii*, *Gemella haemolysins* and *Eubacterium S17* made up ca. 9% each of the planktonic population, however these latter two species were not isolated again during this experiment. *Corynebacterium matruchotii* reappeared on day 21 at 8% of the population, suggesting that it was present throughout at undetectable levels. On day 7, after 2 days of biofilm establishment, veillonella and faecalibacteria were isolated at 10% and 11% each of the whole population, veillonella were not isolated again, however, *Faecalibacterium prausnitzii* was isolated on day 21 in similar proportions of the community. Bifidobacterium comprised 12-15% of the biofilm on days 13 and 21, while actinomyces appeared on day 13 (14%) and decreased by day 21 (9%). By day 21, this oral community had become more diverse with species from 10 genera isolated, including peptostreptococci, gardnerella and clostridia. However, due to malfunctioning

of the water bath, temperature conditions may have varied by this day, making these results ambiguous (Fig. 4.4).

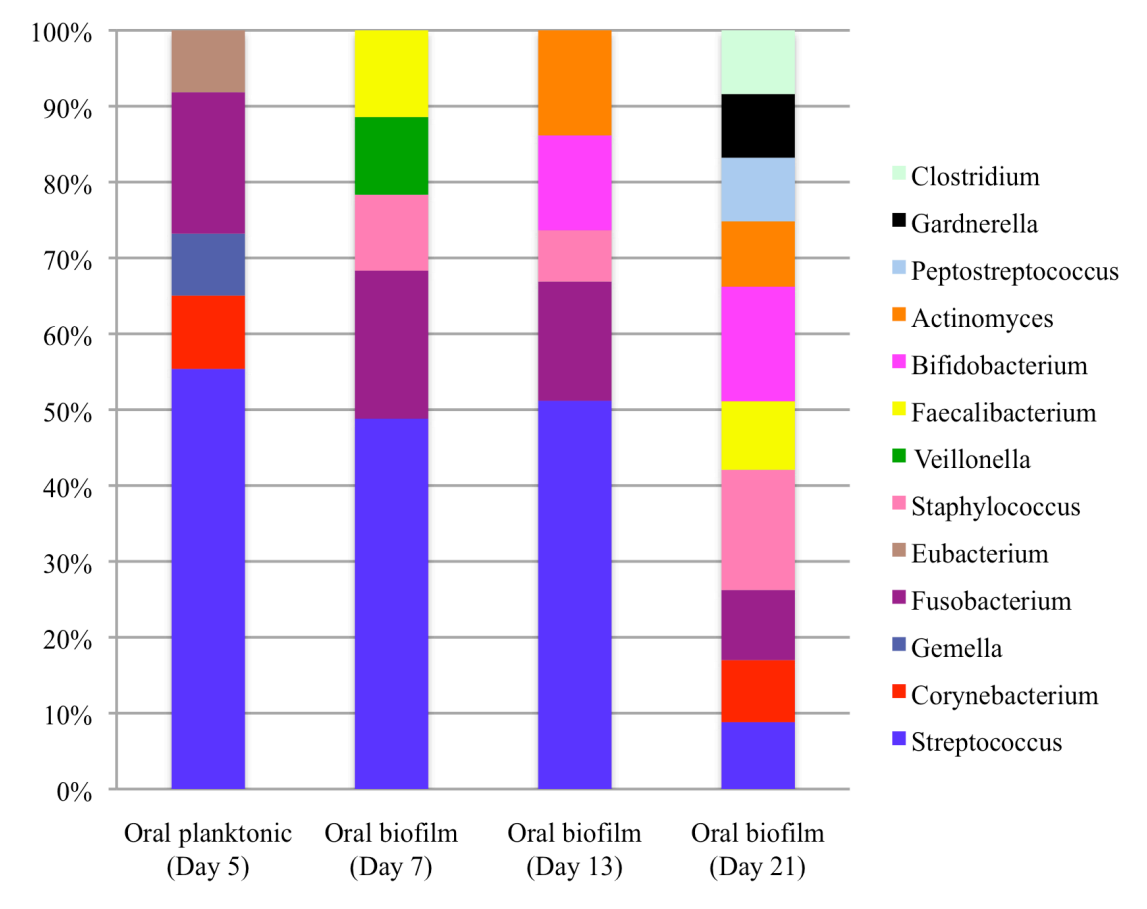


Fig. 4.4: *Global percentages of the oral community in chemostat vessel A (test 1).*

The oesophageal community varied in comparison to that in the oral vessel (Fig. 4.5, Table 4.4). In the planktonic phase, streptococci similarly held the greatest proportion, with the bridge organism, fusobacteria, comprising 24%. Corynebacteria and veillonellas represented ca. 12% each, with the former being isolated only on day 11 in the biofilm after 8 hours of bile acid exposure (9%). Veillonella species were isolated after 2 days of biofilm establishment; however, they were not detected in future

samples. Staphylococci represented the smallest proportion of the planktonic community (4%), and in conjunction with streptococci were the only genera detected in all samples throughout the experiment. *Staph. warneri* was isolated from all samples increasing in abundance throughout, reaching a peak at 21% of the community on day 11 before the start of testing, in conjunction with *Staph. epidermidis*. On exposure to bile acid, this genus suffered a reduction after 8 hours (11%), recovering to 16% by day 12. *Faecalibacterium prausnitzii* and *Rothia denticariosa* were found in low numbers (9% and 7%, respectively) on day 7, and were not detected in future samples. Fusobacteria were isolated from the mucin traps on days 7 and 11 (25% and 22%, respectively), however, after 8 hours of bile acid exposure were undetectable, reappearing after an overnight recovery period (18%).

On day 11, before the start of refluxate testing, species diversity had increased, with staphylococci (21%) appearing to replace streptococci (down to 20%). *Clostridium malenominatum* and *Peptostreptococcus micros* appeared briefly, but were not isolated from future mucin gels. *Prevotella tannerae* comprised 8% of this community, increasing to almost 12% after 8 hours of refluxate exposure; however, this species was not detected subsequently. The oesophageal biofilm community was affected by the exposure to bile acid solution. Streptococci greatly increased in this 8 hour period (20% to 36%), while staphylococci were reduced by 10%. Actinomyces were isolated in this sample period in low numbers (9%), and continued to hold this proportion of the community on day 12. The species *Campylobacter concisus* also appeared after 8 hours of refluxate exposure, becoming the second most predominant species in the mucin gel

microbiota (12%). Campylobacter was only identified in this one sample; however, this could be due to variations in each mucin gel, or due to low culture numbers. The species *Capnocytophaga sputigena* was originally isolated at 0 hour of testing (9%), increasing to 11% of the community after 8 hours of refluxate. By day 12 this organism had increased from $\log_{10}$ 5.8 CFU to $\log_{10}$ 6.9 CFU $disc^{-1}$, however, its percentage in the whole community was reduced (9%). The decreased percentage of this bacterium, despite its increased growth, was due to the appearance of *Propionibacterium acnes* and the pathogenic organism, *Selenomonas infelix* (9% and 8%, respectively).

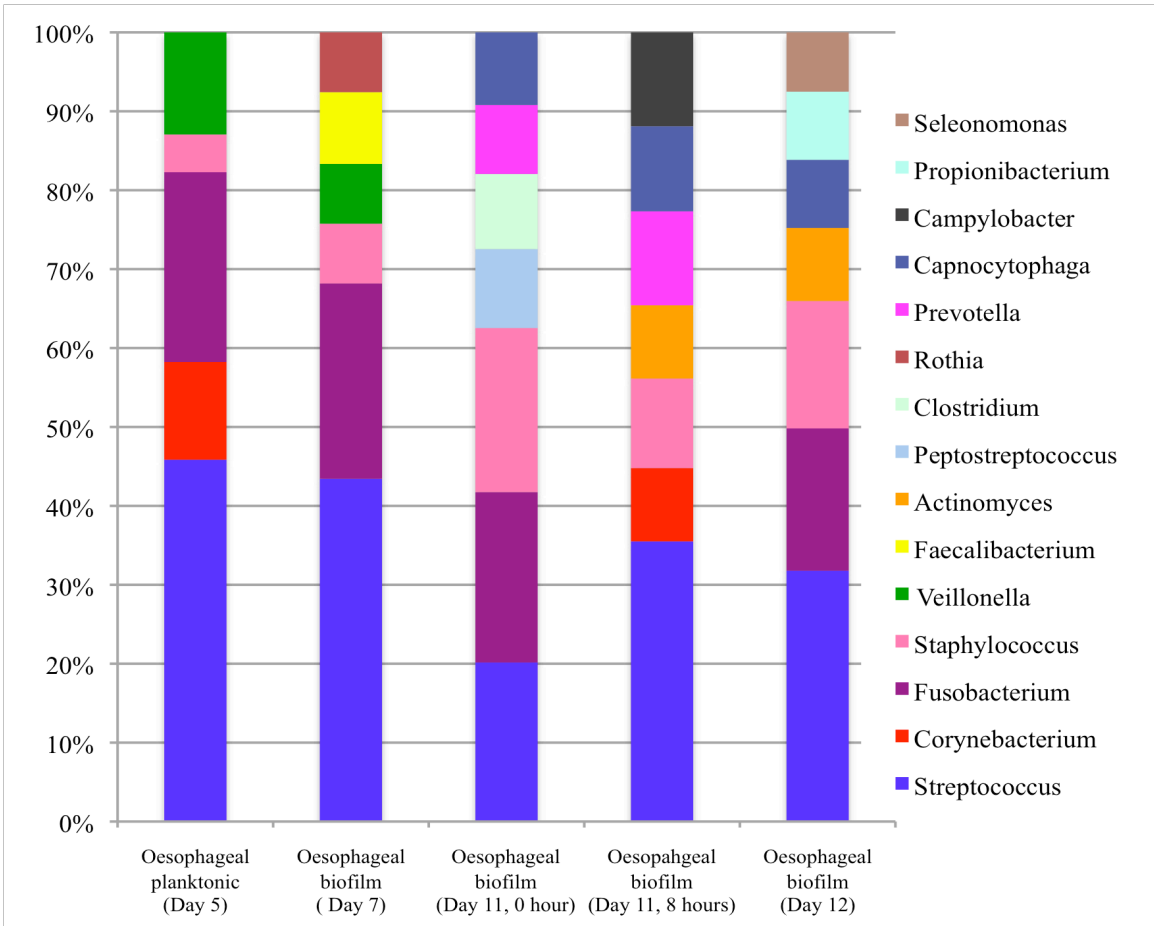


Fig. 4.5: *Global percentages of the oesophageal community in chemostat vessel B (test 1).*

4.3.5 Effect of bile acid exposure on toxin and mucinolytic enzyme production

The effect of a bile acid cocktail on the virulence of oesophageal biofilms was investigated using this continuous culture system. Table 4.5 shows that haemolysin formation increased from 5.9 U before exposure to 43.4 U $\log_{10}$ 8 CFU^{-1} after 8 hours of periodic contact with this solution. Over the following 9 days the level of haemolysin production increased dramatically to 367 U $\log_{10}$ 8 CFU^{-1}, indicating that the toxicity of this community increased with stress due to refluxate, possibly due to an increasingly Gram negative composition. Additionally, the majority of this haemolysin was cell bound, although higher levels of extracellular protein were present by day 20.

Table 4.5: *Formation of haemolytic toxin in the model oesophageal chemostat after exposure to a bile acid cocktail (pH 3)* [a].

Day	Haemolysin	
	Cell-bound	Extracellular
Day 11[b]	5.9 ± 1.7	2.7 ± 0.6
Day 11[c]	43.4 ± 13.0	31.5 ± 1.8
Day 15	88.3 ± 11.0	33.7 ± 2.5
Day 20	367.0 ± 18.3	172.5 ± 51.6

[a] *Results are means ± SD of data obtained in the second experiment with duplicate samples tested, data expressed as units of activity (U) $\log_{10}$ 8 CFU^{-1}.* [b] *0 hour of exposure to bile and acid.* [c] *Following 8 hours of exposure to bile and acid.*

Table 4.6 shows an increase in the activities of a number of enzymes involved in mucin breakdown (β-galactosidase, α-fucosidase, and *N*-acetyl β-glucosaminidase). All three enzymes were increased by day 20, however, very little change was seen in the first 8 hours. After 4 days of exposure (day 15) levels of β-galactosidase had increased from 1.5 to 2.5, and by day 9 reached 8.0 U $\log_{10}$ 8 CFU^{-1}. α-fucosidase levels had a greater increase from 1.2 on day 1 to 13.6 U $\log_{10}$ 8 CFU^{-1} by day 9 of exposure. While *N*-acetyl

β-glucosaminidase showed the greatest increase, rising from 2.8 at 0 hour to 3.6 after 8 hours, furthermore, by day 4 levels were 7.5, increasing to 28.0 U $\log_{10}$ 8 CFU^{-1} on day 9 (Table 4.6). β-Galactosidase activity was found in similar levels both cell-bound and extracellularly, while α-fucosidase had slightly higher cell bound activity. *N*-acetyl β-glucosaminidase was found cell-bound and extracellularly, however, by day 9 this enzyme was found in higher quantities cell-bound, constituting 70% of its total activity.

Table 4.6: *Formation of mucinolytic enzymes in the model oesophageal chemostat after exposure to a bile acid cocktail (pH 3)* [a].

Day	β-Galactosidase		α-Fucosidase		*N*-acetyl β-Glucosaminidase	
	Cell-bound	Extracellular	Cell-bound	Extracellular	Cell-bound	Extracellular
Day 11[b]	1.5 ± 0.0	1.1 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	2.8 ± 0.1	2.8 ± 0.5
Day 11[c]	0.4 ± 0.4	ND[d]	1.0 ± 0.4	ND	3.6 ± 0.5	1.9 ± 0.3
Day 15	2.5 ± 0.1	3.0 ± 0.6	2.6 ± 0.1	2.2 ± 0.3	7.5 ± 0.8	7.2 ± 0.7
Day 20	8.0 ± 3.3	8.4 ± 0.9	13.6 ± 0.7	6.1 ± 2.0	28.0 ± 2.4	11.0 ± 0.9

[a, b, c] *See legend to Table 4.5.* [d] *ND, not detected.*

These results show that with exposure to bile acid, stress leads to an increase in these mucinolytic enzymes, predominantly *N*-acetyl β-glucosaminidase. However, numbers were low, being expressed per 1 x 10^8 CFU, while total cell counts in these mucin gels were between $\log_{10}$ 7.5 to $\log_{10}$ 8.2 CFU $disc^{-1}$ during refluxate testing.

Neuraminidase was not expressed by these cells, data is not shown.

4.4 Discussion

The aim of this research chapter was to design a system which would model the oral and oesophageal microbiotas, allowing *in vitro* investigation of the effect of refluxate on an established oesophageal community. This study represents the first attempt to design a model of the oesophagus for investigation. Initial microbiological results indicate that two distinct communities were established in the oral and oesophageal chemostat vessels, with greater diversity and increased presence of Gram negative species in the oesophageal vessel, particularly following exposure to a bile acid cocktail.

If this model is to be utilised for future research, certain aspects of its design and implementation could be improved. Firstly, the lack of further inoculations may have hindered the establishment of a complete model biofilm. A study by McKee *et al.* (1985), used batch cultures of nine pure isolates to inoculate their chemostat, these cultures were then used to reinoculate the vessel on two subsequent occasions to enhance establishment of the oral community. Consideration of the use of multiple defined batch cultures was considered for this study, however, it was concluded that a truer representation of a normal, healthy and diverse oesophageal microbiota would be obtained through inoculation with fresh saliva from a healthy volunteer. Although this model did largely represent the oesophageal community, key species such as neisseria, lactobacillus and bacteroides were not isolated. The study by McKee and co-workers observed that neisseria only developed in the oral community after the third inoculation (McKee *et al.*, 1985); therefore, further additions of fresh saliva in this present study may have permitted a more complex, diverse and representative community to develop.

The composition of dental plaque varies both spatially and temporally, being modified by cleaning, eating, drinking and general health status (ten Cate, 2006; Signoretto *et al.*, 2010), therefore, the use of fresh saliva may not generate reproducible results. Consequently, for future research, comparison of the community established after multiple inoculations with mixed defined cultures vs. saliva should be investigated, to determine the most reliable and representative method for development of an *in vitro* oesophageal microbiota.

During the initial experiment, increased collection of planktonic samples throughout the 21 days would have provided further information about the development of a steady state in these vessels. During exposure of mucin gels to bile acid, samples should have been taken regularly after day 12, as the mechanical breakdown of this model by day 21 reduced the abundance of data for successive exposures. Additionally, during reflux exposure to the oesophageal vessel, the ability to take control samples of the mucin gels would have provided valuable information about the full effect of bile acid on the community. Control samples could not be utilised to their full potential due to lack of space in the vessel, however, if this model was to be redesigned for future work, larger vessels with a greater capacity for sample collection could be employed. The initial experimental model provided full microbiological data, however, this was a pilot trial to gauge the potential of creating this *in vitro* oesophageal community. Regrettably, haemolytic and mucinolytic enzyme experiments were not carried out in this first run, while the second experiment provided the reverse, therefore, no matched microbiological and enzymatic data is available.

Limited results from this study indicate that two distinct microbiomes established in the oral and oesophageal vessels, with a further shift in composition of biofilms formed on mucin gels. Three bacterial phyla were identified in the oral vessel of test one: Firmicutes, Actinobacteria and Fusobacteria. In these phyla, eight genera of Firmicutes were isolated: *Streptococcus, Staphylococcus, Faecalibacterium, Gemella, Eubacterium, Veillonella, Peptostreptococcus* and *Clostridium*. The Actinobacteria, *Corynebacterium, Actinomyces, Bifidobacterium* and *Gardnerella* were identified with the genus *Fusobacterium* in the third phylum.

Species from five phyla were detected in the oesophageal vessel, both planktonically and in biofilm communities: Firmicutes, Actinobacteria, Fusobacteria, Bacteroidetes and Proteobacteria. In these phyla, seven genera of Firmicutes were isolated: *Streptococcus, Staphylococcus, Faecalibacterium, Veillonella, Peptostreptococcus, Clostridium* and *Selenomonas.* The Actinobacteria, *Corynebacterium, Actinomyces* and *Rothia* were identified, with *Fusobacterium* from its phylum, Fusobacteria. The Bacteroidetes, *Prevotella* and *Capnocytophaga* were detected, while the Proteobacteria *Campylobacter* was also isolated. These same five phyla correspond with the findings in Chapter 2, investigating the bacterial communities found *in vivo* in the oesophagus of 34 patients. Of all genera identified, only capnocytophaga and selenomonas species were not isolated from the clinical study. The genera *Gemella, Eubacterium, Bifidobacterium* and *Gardnerella* were isolated from patients, however, in this chemostat model were only cultured from the oral vessel. Additionally, micrococcus, neisseria and leptotrichia were found in patients, and although not characterised in this study, morphological

observations from the second experimental run indicate that these species may have been present. The genera *Bacteroides*, *Lactobacillus*, *Enterococcus, Lactococcus* and *Arcobacter* were only isolated from patients in the clinical study.

Lactobacillus was increasingly identified in ADC patients (Fig. 2.8), and was proposed to have originated in the gastric microbiota, being regurgitated during reflux disease. The lack of entry of a gastric microbiota into the oesophageal vessel is a limitation of this study, since it was proposed that changes in the oesophageal populations with disease are due to not only the effects of bile and acid in refluxate, but also its microbial content. Total CFU cm^{-2} in control, GORD, BO and ADC patients (Chapter 2), ranged from mean log_{10} 4.0 to log_{10} 8.6 CFU cm^{-2}, while in this oesophageal chemostat model, total counts ranged from mean log_{10} 8.0 on day 2 of biofilm formation, to log_{10} 7.0 CFU $disc^{-1}$ (0.8 cm) after bile acid exposure. Total counts in the planktonic phase reached mean log_{10} 9.7 CFU ml^{-1} in the oesophagus, and mean log_{10} 10.0 CFU ml^{-1} in the oral vessel, similar to previous studies (McKee *et al.*, 1985; Bradshaw *et al.*, 1989). Therefore, although this model is lacking some common oesophageal species, as a first attempt, it appears that a continuous culture system could be used as a model of the oesophageal microbiota, to better understand its composition, and investigate the effects of treatments on these indigenous communities.

Although certain species held similar proportions of the community throughout the initial experiment, variations were found throughout, with some bacteria being temporarily undetectable, reappearing in subsequent mucin gels. These discrepancies

could be due to a number of reasons: effect of primary colonisers, variability in each suspended disc, or low abundance for culture collection. The development of a complex biofilm is dependent on primary, secondary and bridge colonisers such as *Streptococcus*, *Fusobacterium*, *Actinomyces* and *Veillonella* (discussed in Section 1.5.2). In this model, streptococci and fusobacteria were commonly isolated, however, veillonella species were only identified planktonically and on day 2, while actinomyces were detected on days 11 and 12. As seen in oral community development, diverse niches allow development of differing biofilm structures, with variation in respective species composition (Kolenbrander, 2006). The primary colonisers of a surface provide a niche for specific species dependant on their individual metabolic requirements; therefore, in this model each mucin gel may have supported a slightly varied community dependent on its initial colonisers. It is likely, that in the planktonic phase, bacterial aggregates were formed, with streptococci and fusobacteria interacting with a variety of species, possibly undetected by culture due to low abundance. Although medium was continually agitated and mixed, these aggregates were potentially the original colonisers of the mucin platforms, hence, leading to variation in the community structure of each biofilm. Over 700 species are present in the oral cavity, with over half unidentifiable using culture methods (Aas *et al.*, 2005), however, as discussed in Chapter 2, molecular and culture based studies of oesophageal communities do not show the same discrepancies. Nevertheless, it is possible, that many species were present in mucin gel communities in low abundance and were not detected by culture.

Fusobacteria are highly important species in community development, being bridge organisms between primary and secondary colonisers, additionally facilitating association between aerobic and anaerobic species (Bradshaw *et al.*, 1998). In this study by Bradshaw and co-workers (1998), a continuous culture system was set up to investigate the effects of *F. nucleatum* on microbial communities and co-aggregation dynamics. Results showed that this species could bind to many streptococci, actinomyces and lactobacilli, which were also able to coaggregate independently, however, this species also allowed close association between species such as *Neisseria subflava* which poorly aggregated with the above genera. Furthermore, this organism allowed the survival and persistence of a number of anaerobic species, which were reduced on removal of *F. nucleatum* from the system. Representatives of this genera were present throughout this research study, in the oesophageal vessel, excluding the sample taken after 8 hours of bile acid exposure, where they were temporarily undetectable, similar to that found in a previous study of the oral cavity with the antimicrobial agent, chlorhexidine (McDermid *et al.*, 1987). On day 11, where fusobacteria were not recovered, there was a sudden presence of *Campylobacter concisus* and actinomyces. Moreover, in the previous sample, before refluxate exposure, streptococci were drastically reduced, while staphylococci increased in prevalence, and peptostreptococci, clostridia, prevotella and capnocytophaga appeared. Therefore, these two initial colonisers may be very important in community development, leading to disruption of the whole population due to changes in their relative abundances.

Costerton *et al.* (1994) reported that *in vivo* biofilm populations were distinct from their planktonic counterpart, with primary colonisers being selected dependant on their adherence abilities. The metabolic profiles of these founding bacteria determine the successive colonisers of the community (Costerton *et al.*, 1994). A past study by Liljemark *et al.* (1981) utilized hydroxyapatite to measure the adherence potential of streptococci and its coaggregation partners. Results indicated that an increased proportion of streptococci in a streptococci/actinomyces aggregate resulted in continued increase in size of the mass, however, this lead to a lower proportion of non-aggregated bacteria adhering to the surface (Liljemark *et al.*, 1981). Additionally, studies (Liljemark *et al.*, 1981; Costerton *et al.*, 1994) have shown that large aggregates result in a lower number of species adhering to a surface, while the greatest increase in adherence and diversity occurred with smaller aggregates with lower prevalence.

In this study, where the planktonic phase was left to reach steady state for five days before addition of mucin gels, larger aggregates may have formed. This could have a detrimental effect on mucin biofilm composition, due to a limitation in species adherence due to a greater mass of simple aggregates. Golub *et al.,* (1979) found that *Streptococcus sanguinis* was able to bind to saliva within 5 minutes. Therefore, a comparison of varying lengths of time for addition of mucin gels, together with the mode of inoculation, to allow optimum community diversity and representation, should be carried out for future models.

Results from the second chemostat model indicate that the biofilm populations did not have haemolytic activity at the start of experimentation (Table 4.5). Levels of haemolysin were extremely low on day 11, although no results were taken before this time, samples taken in the following three time periods indicate a stress response by the microbiota, or selected species within. Data represents a 12 - 15% increase per day, however, within the first 8 hours, after the first shock bile acid exposure, levels of activity increased by 86%. The lack of microbiological data for this second experiment do not allow a correlation of haemolytic activity with the presence of specific species. However, the increase in Gram negative species, such as *Campylobacter, Selenomonas*, *Neisseria*, *Leptotrichia* and *Capnocytophaga*, proposed through morphological observation, could be associated with this increased haemolytic activity. Studies with *Campylobacter concisus* have revealed the presence of a membrane bound phospholipase A (PLA) protein (Istivan *et al.*, 2004, 2008). This species additionally seems to secrete iron regulated cytotoxin, which could explain the increase in both cell bound and extracellular activity with stress. *H. pylori* has a similar PLA, regulated by the *pld*A gene, which is involved in colonisation of the gastric mucosa and the related damage to this organs tissue (Dorrell *et al.,* 1999). Bile salts in high concentration can affect bacterial membranes, therefore altering their permeability; this can induce DNA damage and lead to oxidative stress, possibly resulting in increased haemolysis (Begley *et al.*, 2005). Additionally, the exposure of bile salts to *C. jejuni* resulted in an increased expression of CiaB protein (Malik-Kale *et al.*, 2008), possibly pili, and the flagellin, *flaA*, involved in motility and virulence (Begley *et al.*, 2005).

Although data is limited, initial analysis indicates that with reflux exposure, microbiota composition is altered, becoming more diverse and Gram negative, correlating with data from Chapters 2 and 3. Furthermore, this exposure results in an increased stress response, with greater virulence potential and production of mucinolytic enzymes, illustrated by the amplified expression of both cell bound and extracellular haemolysins. This chemostat, modified and improved, could prove a useful *in vitro* model to enhance understanding of the oesophageal microbiota, and the effects of environmental factors. Bacterial isolates from this model may also be used to investigate their effects on epithelial cells using cell culture, providing information on genetic, epigenetic and inflammatory responses to these control and stressed populations.

Chapter 5

Co-culture studies investigating the involvement of bacteria in cancer using oesophageal cell lines

5.1 Introduction

The human microbiome is part of a highly complex system which has evolved with its host to be mutually beneficial, balancing its interactions with the epithelia to maintain normal host physiology. This ecosystem can also comprise opportunistic and pathogenic species, which under unstable conditions, such as changes in host cell activity or the immune response, can leave the host susceptible to infection and disease. Cell lines for most tumour types are available, and are utilised extensively in co-culture with bacteria to measure responses of eukaryotic cells to infection. Cell line co-culture experiments can allow a deeper understanding of host responses to a specific organism, in this case, *Campylobacter concisus*, however, as discussed previously, it is unlikely that pathogenicity is caused wholly by the presence of a single species. Therefore, rather than a single organism, the whole biofilm it is complexed with should be investigated to better understand mechanisms of disease.

Little research has been carried out on the host response to *Campylobacter concisus* using cell lines, however, *C. jejuni* has been extensively studied. MacCallum and co-workers (2006) exposed a range of colon cancer cell lines and patient tissues to this organism. *C. jejuni* caused an increase in IL-8 production, especially in primary tissue, where a full immunological network was present. A further study by Zheng *et al.* (2008) exposed the colon carcinoma cell line, T84, to *C. jejuni* and *C. coli*. These species required CDT and flagella functionality to induce an inflammatory response, indicated by IL-8 production dependent on TLR activation of the NF-kappa B signalling pathway. Incubation of clinical *C. concisus* strains with CHO cells resulted in cytolytic

destruction of cells with vacuolation (Istivan *et al.*, 2004) due to the haemolytic effects of phospholipase A, encoded by the gene, *pldA* (Istivan *et al.*, 2008). Results indicate that *Campylobacter* species, especially *C. concisus*, are present increasingly with oesophageal disease (Macfarlane *et al.*, 2007; this study) and may increase the haemolytic activity of the biofilm due to bile acid stress (Chapter 4). This chapter's research was designed to take a preliminary look at the effect of this species on the oesophagus using a range of cell lines.

Results from Chapters 2 and 3 indicate that there is not a single aetiologic agent in oesophageal disease progression, with a shift in the whole microbiota being indicative of disease. Pei's group in New York published a small pilot study investigating the effects of *Streptococcus mitis* and *Veillonella atypica* on the macrophage cell line THP-1 (Lu *et al.*, 2006). Incubation of these heat inactivated bacteria with the cell line for 72 or 96 hours resulted in increased expression of COX-2 and NQ01; both of which are involved in inflammation and protection from oxidative and toxic stresses, such as bile acid reflux. Additionally, when in combination with acid, COX-2 expression was further amplified, while NQ01 was weakened. It is not fully understood why this study used only the cell line THP-1, and not also an oesophageal cell line such as OE19 or FLO-1. Nevertheless, this preliminary study indicates that opportunistic bacteria from the oesophagus may promote the inflammatory status of reflux oesophagitis, especially when exposed to acid.

This chapter aimed to not only investigate the effects of *Campylobacter concisus* on a range of oesophageal cell lines, but to carry out preliminary experiments with biofilm samples taken from the *in vitro* model of the microbiota established in Chapter 4. Fortunately, results from this chemostat modelling indicate that a community representative of that *in vivo* was developed, with the microbiological effects of refluxate exposure simulating the clinical development of GORD to ADC.

Multiple cell lines of oesophageal origin were employed in this study to fully represent the progression of disease. Cells derived from Barrett's-associated ADC and squamous cell carcinoma were used along with the newly developed hTERT immortalised Barrett's cell lines, CP-A, -B and –D (Palanca-Wessels *et al.*, 2003). The CP-A strain contains wild-type p53, while the other two have a loss of heterozygosity and mutation in this gene. This study of immortalised Barrett's cell strains indicated that although telomerase can reduce genetic instability, levels of chromosomal instability persist, allowing continued neoplastic progression.

A number of recent studies have characterised the p53, COX-2 and Ki-67 status of the metaplastic-dysplastic sequence of BO and ADC. With progression from a normal oesophagus to ADC, over-expression of the mutated tumour suppressor gene, p53, has been observed, with increasing expression correlated with metastasis and invasion category (Feith *et al.*, 2004; Murray *et al.*, 2006; Binato *et al.*, 2009). COX-2 and Ki-67, a marker of proliferation, were both progressively amplified with disease severity (Feith *et al.*, 2004; Binato *et al.*, 2009). It has also been proposed that the most significant

increase in COX-2 expression occurs in the early stages of disease between squamous epithelia and Barrett's metaplasia, being associated with the inflammatory response (Ling *et al.*, 2007).

In this study, a range of oesophageal cells were exposed to both *Campylobacter concisus* and chemostat biofilms (Chapter 4). Measurements for adhesion and invasion of bacteria and cytotoxicity were performed, while cell samples were also taken for western blot and immunohistochemistry analysis. These assays were carried out in the final stages of the project as a highly preliminary experiment, with antibodies that were easily accessible at the University. Western blot analyses of the readily available p53, p21 and COX-2 proteins were performed. Samples were also sent to the Histopathology unit for standard Ki-67 staining.

This research hypothesised that with exposure of a range of oesophageal cells to campylobacter and a representative microbiota, expression of these host response proteins, and in particular COX-2, would be increased. Additionally, due to the increased virulence of the oesophageal biofilms exposed to bile acid, invasion potential and cytotoxicity would be enhanced.

5.2 Materials and methods

5.2.1 General cell culture

The Barrett's-associated adenocarcinoma cell line FLO-1 was a kind gift of Dr R Fitzgerald (Department of Oncology, Hutchison Medical Research Council Research Centre, University of Cambridge). Stocks of these FLO-1 cells were maintained in Dulbecco's Modified Eagle Medium containing L-glutamine (580 mg L^{-1}) and D-glucose (1000 mg L^{-1}), supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (Gibco/Invitrogen, Paisley, UK). Squamous cell carcinoma OE21, and oesophageal adenocarcinoma, OE19, were maintained in Roswell Park Memorial Institute (RPMI) 1640 media containing identical supplements. Pre-neoplastic cell lines of Barrett's oesophagus were established from well-characterised patient biopsies using human telomerase for immortalisation (Palanca-Wessels *et al.*, 2003). CP-A is a non-dysplastic cell line, while CP-B and -D are cells of high-grade dysplasia origins. These Barrett's cell lines were a kind gift of Dr Peter Rabinovitch (Fred Hutchison Cancer Center, Seattle WA, supplied by Dr Rebecca Fitzgerald). These Barrett's cell lines were maintained in Keratinocyte Serum-free medium with supplements of 30 mg L^{-1} Bovine Pituitary Extract and 0.2 μg L^{-1} recombinant Epidermal Growth Factor with 1% penicillin/streptomycin (all Gibco/Invitrogen, Paisley, UK).

All cells were grown as adherent monolayers in T25 and T75 (25 cm^2 and 75 cm^2) cell culture flasks (Corning, High Wycombe, UK) and incubated at 37^oC in a 5% CO_2 humidified atmosphere. Stock cells were grown to 90-95% confluency, at time of

passage growth medium was removed, cells were washed with PBS, and treated with warmed trypsin solution (both Gibco/Invitrogen, Paisley, UK) before incubation at 37^{o}C for 2-10 min to facilitate removal of cells. Trypsin was inactivated by the addition of RPMI 1640 or DMEM supplemented with FCS. Cells were seeded into fresh flasks at a dilution of 1 in 10 of the neutralised cell suspension. After trypsinisation to remove the Barrett's cells, 250 mg L^{-1} soybean trypsin inhibitor (STI) was utilised for neutralisation.

Frozen stocks of all cell lines were maintained in their respective medium supplemented with 10% FCS and 10% dimethyl sulphoxide (DMSO), and snap frozen with liquid nitrogen before storage at -80^{o}C.

5.2.2 Bacterial strains

Campylobacter concisus CCUG 34767 (Culture Collections, University of Goteborg, Sweden), *Campylobacter concisus* NWBO1, a strain isolated from a GORD patient in this study, and *Campylobacter concisus* NWBO2, isolated from a BO patient, were kept anaerobically at 37^{o}C on Columbia blood agar plates. NWBO1 showed large haemolytic zones on this media, indicating a highly virulent strain, which was utilised for further testing.

5.2.3 Co-culture assays with Campylobacter concisus

All cell lines were grown to confluence in 75 cm^2 TC flasks, and seeded into 12 or 24 well plates to adhere overnight. Cells were counted using 1% trypan blue and a haemocytometry chamber. Cells were trypsinised as above, and neutralised with their

respective media before addition of 2 ml cell culture media and 10 ml plain medium (1 ml per well) to 12 well plates, and 0.5 ml per well in 24 well plates.

Co-culture experiments were performed with fresh *Campylobacter concisus* growth. Plates with full growth were swabbed into 2 ml WC broth, spun down and resuspended in 10 ml of appropriate culture media for each cell line. At the start of co-culture, spent medium was aspirated from each well, before addition of 1 ml (12 well plate) and 0.5 ml (24 well plate) bacteria inoculated culture media. One plate of full campylobacter growth provided an approximate multiplicity of infection (MOI) of 1:100. Table 5.1 below details the co-culture experiments carried out with these cell lines.

Table 5.1: *In vitro co-culture experiments with Campylobacter concisus, detailing conditions and tests carried out.*

Cell line	Campylobacter	Time points	Tests
OE19	CCUG[a], NWBO1[b], NWBO2[c]	9 h	Cytotoxicity
FLO-1	CCUG, NWBO1, NWBO2	9 h	Cytotoxicity
FLO-1	NWBO1	3, 6, 9, 12, 24 h	Cytotoxicity, invasion, adhesion, western blot
CP-B	NWBO1	3, 6, 9, 12, 24 h	Cytotoxicity, invasion, adhesion, western blot
CP-A	NWBO1	24 h	Cytotoxicity, invasion, adhesion, western blot
CP-D	NWBO1	24 h	Cytotoxicity, invasion, adhesion, western blot

[a] CCUG 34767, Campylobacter concisus isolate. [b] NWBO1, Campylobacter concisus isolate from GORD patient (Chapter 2). [c] NWBO2, Campylobacter concisus isolate from BO patient.

Cytotoxicity

At the specific time point for each experiment, media were removed and cells washed 5 times in 1% sterile PBS, removed with trypsin/EDTA and neutralised with respective media/STI (Barrett's cells). Cells were counted using Trypan blue and a haemocytometer for analysis of viability.

Invasion

Bacteria were incubated for the set time periods (Table 5.1) on cell monolayers to facilitate invasion. At set time points, media was aspirated, and cells were washed 5 times with sterile 1% PBS to remove any unbound organisms, before incubation for a further two hours with 100 μg ml^{-1} gentamicin to kill extracellular adherent campylobacters. Cells were removed via trypsinisation, homogenised, serially diluted and spread on Columbia blood agar plates (100 μl) for bacterial counts ($37^{o}C$, 2 day anaerobic growth).

Adhesion

Epithelial cells were grown to confluence on glass coverslips in the 12 or 24 well plates. Non-adherent bacteria were aspirated from the cell monolayer and remaining unbound bacteria were removed by five washes with sterile 1% PBS. Cells were fixed for 5-10 min with ice-cold 70% methanol, and cover slips were removed for visualisation. Co-cultures were stained with 50 μl carbol fuchsin for identification of Gram negative campylobacters. Adherent bacteria were then viewed by phase contrast light microscopy.

Oncogenic response assays

Culture media were removed, cells washed 5 times with sterile 1% PBS and the monolayer was trypsinised for removal. Cells were centrifuged at 1000 *g* for 5 min and snap frozen under liquid nitrogen for storage at -80^{o}C.

For all assays, duplicate controls with plain culture media were used to verify changes induced by *Campylobacter concisus*.

5.2.4 Co-culture assays with chemostat samples

All cell lines were grown to 90 – 100% confluence in 75 cm^2 TC flasks, and seeded into 24 well plates (Section 5.2.3) to adhere overnight before addition of chemostat media. Cells were counted using 1% trypan blue and a haemocytometry chamber.

Co-culture experiments were performed with fresh biofilm samples taken from the oesophageal chemostat vessel (Section 4.2.2). Mucin traps were removed from the vessel and biofilms were added to 2 ml peptone water, homogenised, centrifuged at 1000 *g* for 1 min before resuspension in the appropriate cell culture medium (0.5 ml per well). Homogenisation of the colonised agar allowed the aggregated bacteria to be separated and dispersed. Respective media for each cell type was used, with control experiments run with plain culture medium. Mucin traps from the oesophageal chemostat were removed on day 17, six days after starting refluxate exposure (Table 4.1). In the first chemostat run, an exposed biofilm sample was tested with OE21 and FLO-1 cells, while in the second experimental run, both an exposed and control mucin

biofilm were co-cultured with FLO-1 and CP-D cell lines. MOI was dependant on CFU per mucin trap. Table 5.2 below details the experiments and methods carried out for multi-species co-culture.

Table 5.2: *In vitro co-culture experiments with chemostat samples, detailing conditions and tests carried out.*

Cell line	Biofilm	Time points	Tests
OE21	Test 1, exposed biofilm [a]	3, 6, 9, 12, 24 h	Cytotoxicity, invasion, adhesion, western blot
FLO-1	Test 1, exposed biofilm	3, 6, 9, 12, 24 h	Cytotoxicity, invasion, adhesion, western blot
FLO-1	Test 2, control [b] and exposed biofilm	6, 12 h	Cytotoxicity, invasion, adhesion, western blot
CP-D	Test 2, control and exposed biofilm	6, 12 h	Cytotoxicity, invasion, adhesion, western blot

[a] Chemostat biofilms from the oesophageal vessel. [b] Chemostat biofilms from the oesophageal vessel during test 2, unexposed control taken alongside exposed biofilm.

Cytotoxicity

At designated time points, media was removed and cells were washed 5 times in 1% sterile PBS, removed with trypsin/EDTA and neutralised with respective media/STI (Barrett's cells). Cells were counted using Trypan blue and a haemocytometer for % viability. During the second experimental run, cells had died by 12 hours, and all samples were taken at this point.

Adhesion

Epithelial cells were treated as above (Section 5.2.3, Adhesion). However, co-cultures were viewed with propidium iodide (PI) stain to visualise dead cells. 50 µl (diluted 1:500 in dH_2O) propidium iodide (*BacLight* Viability stain, Molecular Probes Europe)

was added to cells and left for 15 min in a dark room before visualisation with confocal laser scanning microscopy (CLSM). Citifluor (Citifluor, Ltd., London, United Kingdom) was used as a mounting medium, and the slides were visualized with a Nikon Eclipse E800 upright microscope attached to a Nikon PCM 2000 confocal system. Images were captured and overlaid with C-Imaging software (Compix, Inc., Cranberry Township, PA).

Invasion and oncogenic response assays

Methods for invasion assays, acquisition and storage of cells for oncogenic analysis were carried out as described above for co-culture with campylobacters (Section 5.2.3). For measurement of invasion WC blood plates were utilised.

5.2.5 Western blot analysis of cell co-culture samples

Oesophageal epithelial cells co-cultured with campylobacter strains and chemostat biofilms were assessed for changes in expression of p53, p21 and COX-2. Cell pellets were washed in 1% PBS and centrifuged at 1000 *g* for 5 min. Pellets were then prepared with 50 mM Tris pH 8, 150 mM NaCl, 0.1% EDTA, 1% Igepal (Sigma) and a protease inhibitor cocktail (Roche). This preparation was heated at 100^{o}C for 10 min and vortexed for resuspension. Proteins and marker (PageRuler Plus, Fermentas, UK) were separated by SDS–PAGE (NuPAGE 12% Bis-Tris gel with 5% MOPS) at 100 V. The gel was then removed and a membrane sandwich assembled with a nitrocellulose blot. This was placed in the transfer tank with PVDF transfer buffer (10% Tris/glycine, 20% methanol, milliQ water). The transfer protocol was run overnight at 12 V/25 mA. This

membrane was then western blotted using standard conditions and appropriate primary and secondary antibodies (Table 5.3).

Table 5.3: *Antibodies and respective secondary HRP antibodies utilized for western blot analysis of co-cultured oesophageal cell lines.*

Antibody	Dilution [a]	Size (kDa)	Secondary HRP antibody [b]	Source
β-actin	1:1000	42	Anti-mouse	Sigma Aldrich, UK
p53 (DO-1) [c]	1:1000	53	Anti-mouse	University of Dundee
p21	1:1000	21	Anti-rabbit	Santa Cruz, CA, USA
COX-2	1:1000	72	Anti-mouse	Caymen Chemicals, MI, USA

[a] Antibodies diluted in 5% milk buffer. [b] Secondary antibodies, diluted 1:2000 with 5% milk buffer, purchased from DAKO, Denmark. [c] p53, kind donation of Professor Sir David Lane, Surgery and Molecular Oncology, Ninewells Hospital and Medical School, University of Dundee.

The membrane was removed and cut at 50 kDa, before blocking for 1 hour with milk buffer (5% non-fat dry milk, 0.1% Tween-20, 1% PBS). After 1 hour, buffer was removed and 10 ml fresh milk buffer was added with 10 µl antibody, this was left to shake for 1 hour before removing and washing 3 times with milk buffer (3 min each). Secondary antibody (10 µl) was added with 10 ml milk buffer and incubated for 40 min, washed 3 times with milk buffer and twice with PBS/Tween-20 (0.1%). The blot was then dried and 500 µl each of chemiluminescent reagents A and B (ECL, GE Healthcare Life Sciences, UK) were added for 2 min before development in a dark room.

Image J software (NIH, USA) was utilised to measure the density of each band, allowing semi-quantitative densitometry and therefore, relative levels of protein for each sample.

5.2.6 Ki-67 immunohistochemistry

Duplicate samples of those measured for protein expression with western blots were also sent to the Histopathology Unit, Ninewells Hospital and Medical School for MIB-1 staining of Ki-67 proliferation using standard techniques.

5.2.7 Statistical analysis

Due to the preliminary status of these experiments, duplicate tests could not be carried out, and there was insufficient data for detailed statistical analysis. Results were compared using unpaired Student's t-tests, with a P value of <0.05 denoting statistically significant variations in CFU ml^{-1} and viable eukaryotic cell counts with each cell type and test condition. Analyses were carried out using Prism 4.0 Statistical Package (Section 2.2.5). No statistically significant results were found (P values not shown).

5.3 Results

5.3.1 Co-culture with Campylobacter concisus

An initial experiment with FLO-1 and OE19 cells in co-culture was performed to investigate cytotoxicity. These cell lines were exposed to three *Campylobacter concisus* strains: CCUG 34767 (human isolate from a heart ventricle), and two isolates from the patient study (Chapter 2): NWBO1 from GORD, NWBO2 from BO biopsies. The two cell lines were exposed to these strains for nine hours, with the results shown in Figs. 5.1 and 5.2. OE19 cells were most affected by NWBO1, and least by NWBO2 (Fig. 5.1), while the opposite was found for FLO-1 cells (Fig. 5.2). Duplicate samples were taken for each, however, numbers were almost identical, with little margin of error observed.

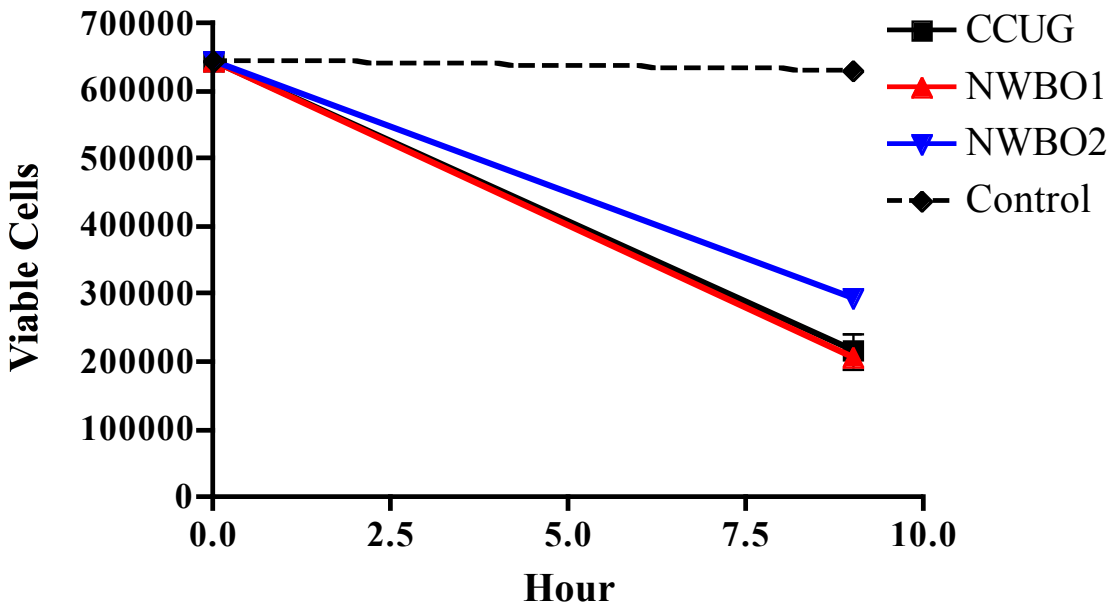


Fig. 5.1: *Counts of viable cells over 9 hours of co-culture with three campylobacter strains (CCUG 34767, NWBO1 and NWBO2) with the cell line, OE19. Control, uninfected (black).*

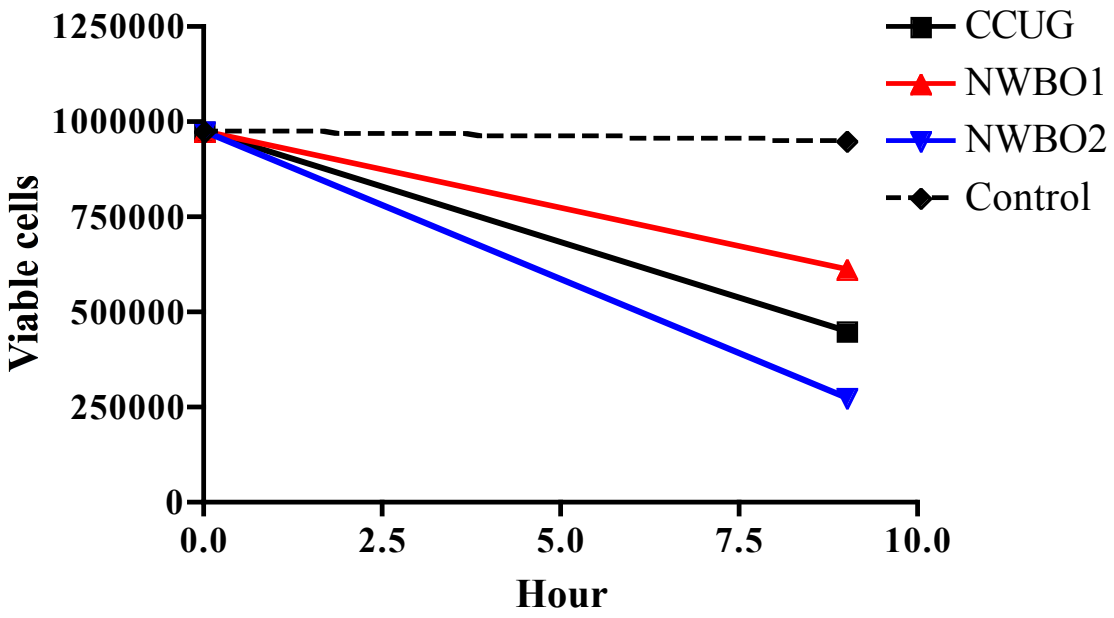


Fig. 5.2: *Counts of viable cells over 9 hours of co-culture with three campylobacter strains (CCUG 34767, NWBO1 and NWBO2) with the cell line, FLO-1. Control, uninfected (black).*

Following these two initial experiments, FLO-1, CP-A, CP-B and CP-D cell lines were exposed to NWBO1. All four experiments show that as invasion of bacteria increased, cell viability was reduced, being almost completely apoptosed by 24 hours (Figs. 5.3 to 5.6). CP-A and CP-D assays were carried out at a later date than FLO-1 and CP-B, with samples only taken at 24 hours, compared with 3, 6, 9, 12 and 24 hours.

Co-culture of NWBO1 with CP-B Barrett's oesophageal cells (Fig. 5.4) revealed a sudden reduction in cell viability from 1.8 x 10^5 to 4.1 x 10^4 cells in the initial 6 hours, decreasing towards zero by 24 hours. Interestingly, no bacteria were found to invade within the first 3 hours, dramatically increasing to ca. 3000 CFU ml^{-1} by 6 hours, in conjunction with this rapid cell death (Fig. 5.4). However, after 6 hours, bacterial CFU decreased, possibly due to a diminishing number of cells for invasion. FLO-1 cells also

showed a rapid decrease in viability, especially in the first 3 hours. For FLO-1 and CP-B assays, levels of bacterial CFU were undetectable at 24 hours, with further dilution plates required.

CP-A and CP-D cells required little invasion of *Campylobacter concisus* for apoptosis, with 100-200 CFU ml^{-1} detected after 12 hours of exposure, compared with 4000 CFU ml^{-1} in FLO-1 cells. CP-D cells had a 63% survival rate compared with 12-14% in FLO-1, CP-A and CP-B cells.

In FLO-1 cells, *C. concisus* NWBO1 led to an increased abundance of p53 protein, while the opposite was observed in the Barrett's oesophagus cell line, CP-A (Figs. 5.16 and 5.17, columns 1, 2, 5 and 6). Taken with the results of β-actin in FLO-1, where cell density was higher in the control sample (Fig. 5.13), this increased expression after exposure was presumably more striking. Western blot analysis showed that CP-A is devoid of COX-2 protein. Interestingly, CP-B and CP-D had a large reduction in COX-2 abundance, in contrast to a dramatic increase in FLO-1 cells (Figs. 5.14 and 5.15).

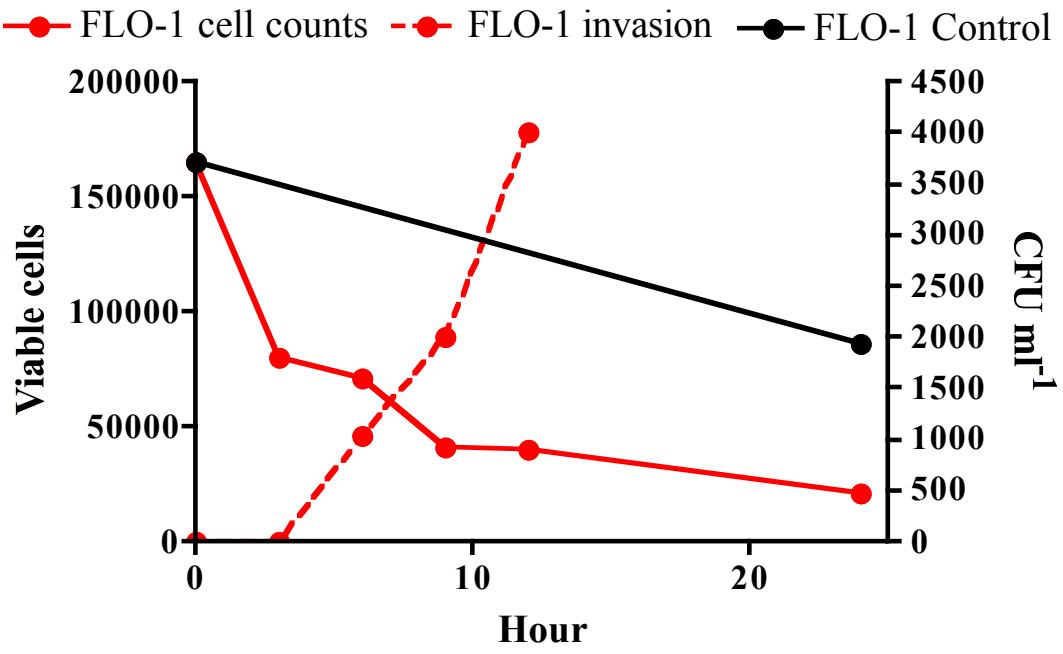


Fig. 5.3: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with Campylobacter concisus NWBO1, isolated from a GORD patient (Chapter 2) with the cell line, FLO-1. Control, uninfected (black).*

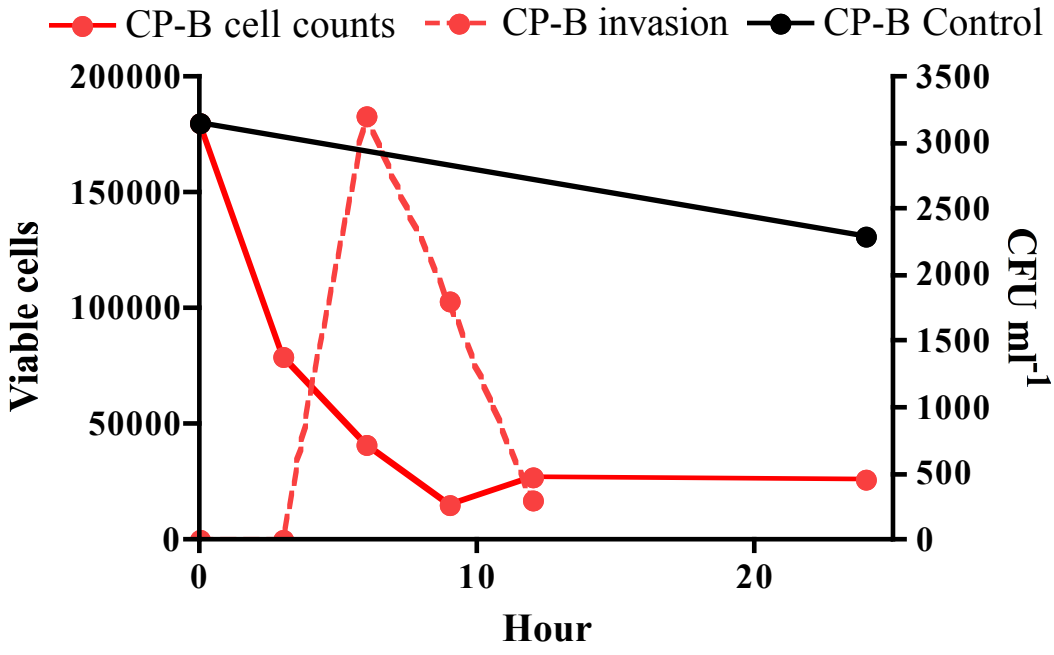


Fig. 5.4: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with Campylobacter concisus NWBO1, isolated from a GORD patient (Chapter 2) with the cell line, CP-B. Control, uninfected (black).*

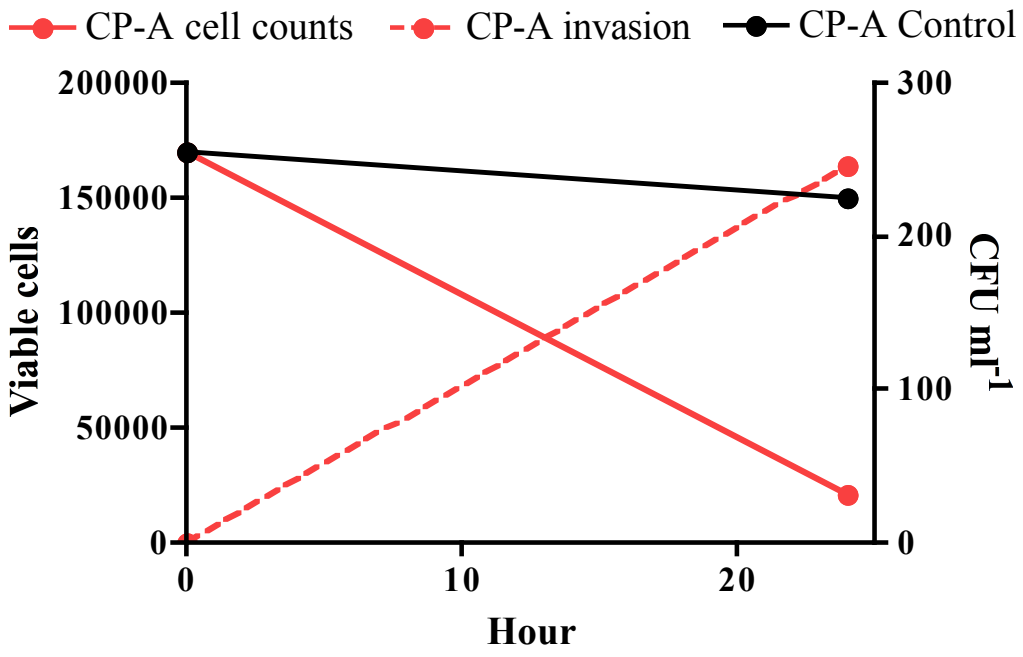


Fig. 5.5: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with Campylobacter concisus NWBO1, isolated from a GORD patient (Chapter 2) with the cell line, CP-A. Control, uninfected (black).*

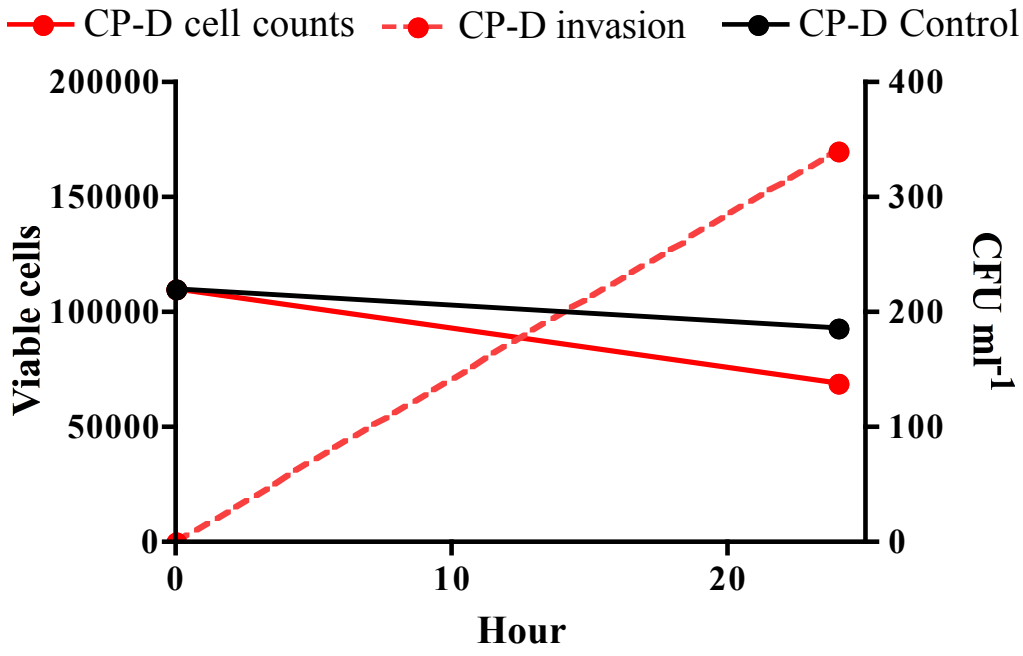


Fig. 5.6: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with Campylobacter concisus NWBO1, isolated from a GORD patient (Chapter 2) with the cell line, CP-D. Control, uninfected (black).*

Fig. 5.7 shows campylobacters adhering to the surface of cells in small aggregates with an area of cell deterioration where bacteria appear to be invading.

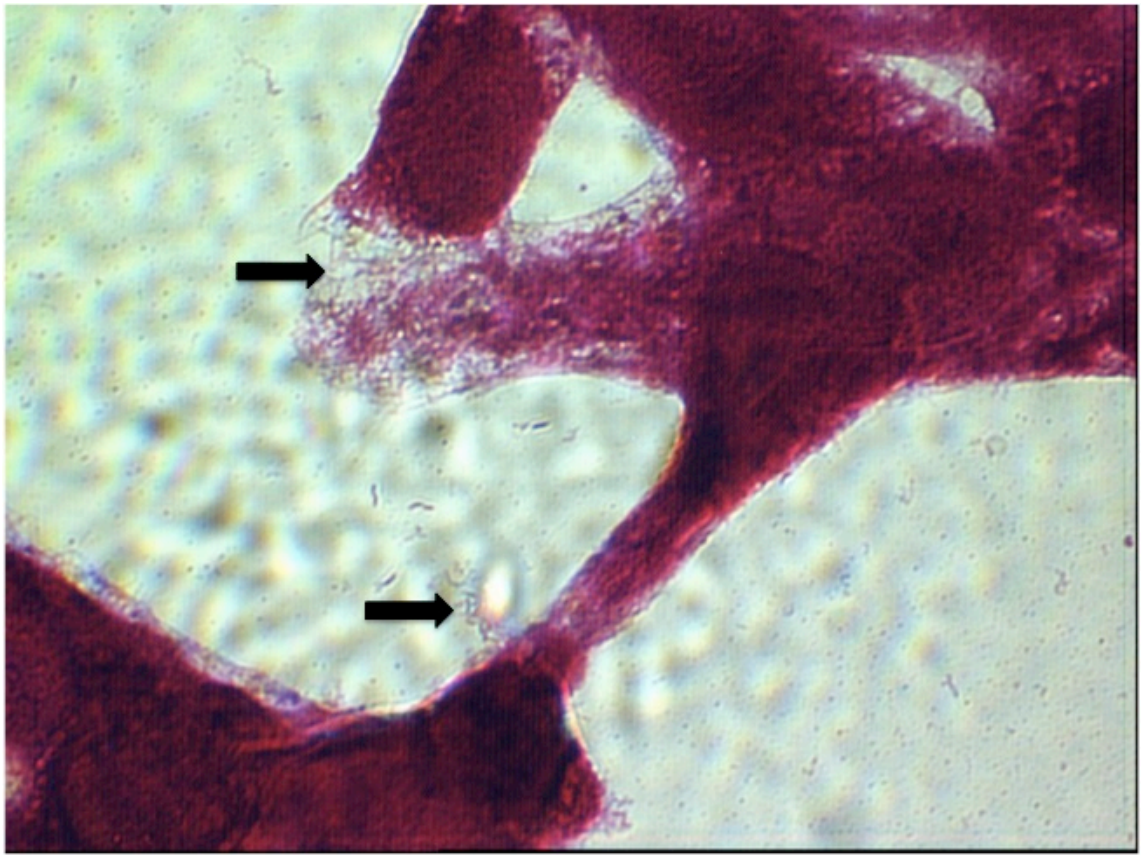


Fig. 5.7: *Image of Campylobacter concisus, NWBO1, co-cultured with CP-B, highly dysplastic Barrett's cells. Black arrows show two areas of invasion, with aggregates of thin curved rods at the surface of cells, and an area of cell disruption and degradation. Eukaryotic and bacterial cells were stained with carbol fuchsin.*

5.3.2 Co-culture with oesophageal chemostat biofilms

Figures 5.8 and 5.9 give the results for the first chemostat run, with OE21 cells (Fig. 5.9) being most effectively invaded and apoptosed compared with FLO-1 cells. However, by 12 hours, although bacterial CFU was considerably lower for FLO-1 cells (10,000 vs. 250,000 CFU ml^{-1}), similar live eukaryotic cells were collected. Therefore, although these columnar cells may be more resilient to bacterial invasion, compared with their squamous counterpart, a lower infection level was required to initiate

apoptosis, with no living cells detected by 24 hours. At this time point, OE21 cells had a bacterial count of 1 x 10^6 CFU ml^{-1}, thus, in the first 9 hours bacterial quantity was undetectable on this scale (20,000 CFU ml^{-1}).

Figures 5.10 and 5.11 show results of co-culture with CP-D and FLO-1 cells respectively, in the second chemostat experiment. At 6 hours, FLO-1 cells had a lower internal CFU ml^{-1} than CP-D, both with control and exposed biofilms. At this time point, there was a 28% survival rate in CP-D compared with 70% in FLO-1 cells, while in the exposed biofilm assay survival was 12% vs. 58%, respectively. By 12 hours, control bacteria had invaded to similar CFU ml^{-1} in both cell lines, while the exposed biofilms had a much greater infection level, most predominantly in FLO-1 cells. By 12 hours, all cells were dead, regardless of treatment; however, CP-D cells underwent apoptosis more readily. Results show that in both co-culture experiments, the exposed biofilm had greater ability to invade and cause cell death. Images of cells adhered to coverslips were taken using propidium iodide dead stain. Fig. 5.12 shows FLO-1 and OE21 cells at 3 (A, B) and 12 (C, D) hours, respectively. Bacterial cells are difficult to distinguish as eukaryotic cells were also stained with the dye.

No p53 or COX-2 protein was detected for CP-D cells exposed to chemostat samples. Conversely, expression of both p53 and COX-2 proteins was dramatically increased in FLO-1 cells (Figs. 5.15 and 5.17, columns 9 and 11) after 6 hours, especially after exposure to the bile acid stressed biofilm.

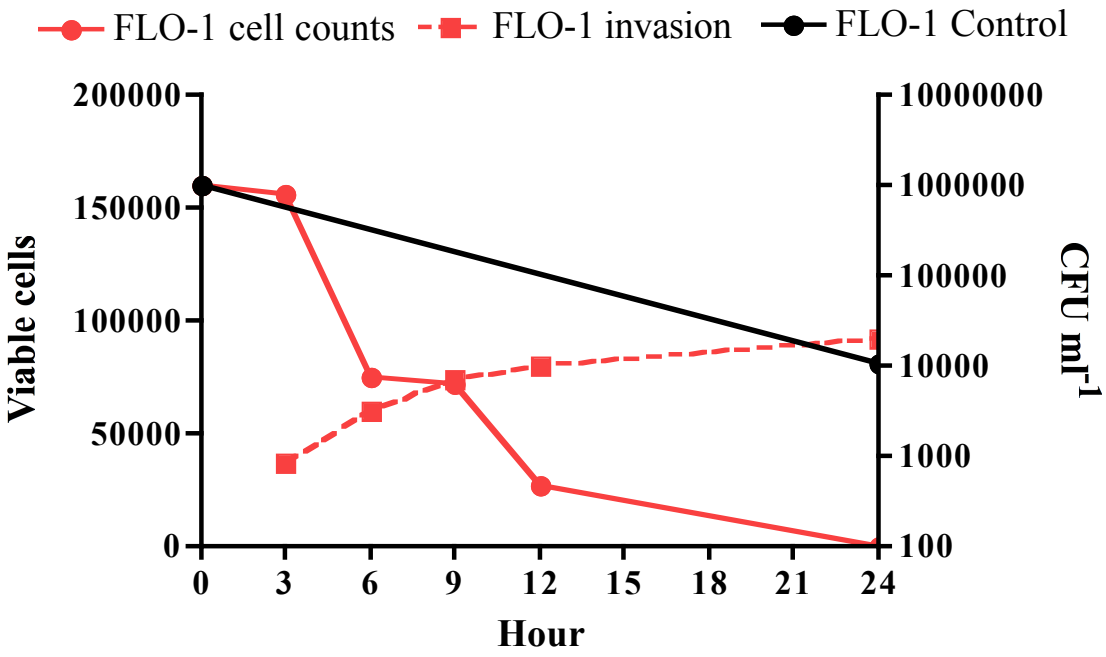


Fig. 5.8: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with a biofilm community removed from the initial oesophageal chemostat with the cell line, FLO-1. Control, uninfected (black).*

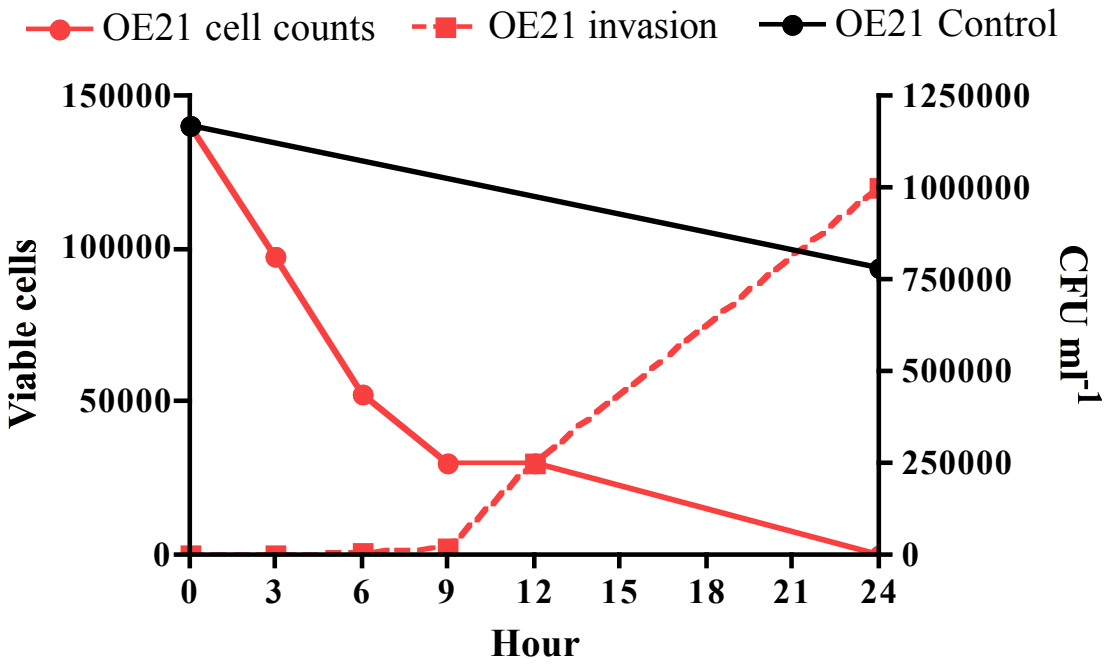


Fig. 5.9: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with a biofilm community removed from the initial oesophageal chemostat with the cell line, OE21. Control, uninfected (black).*

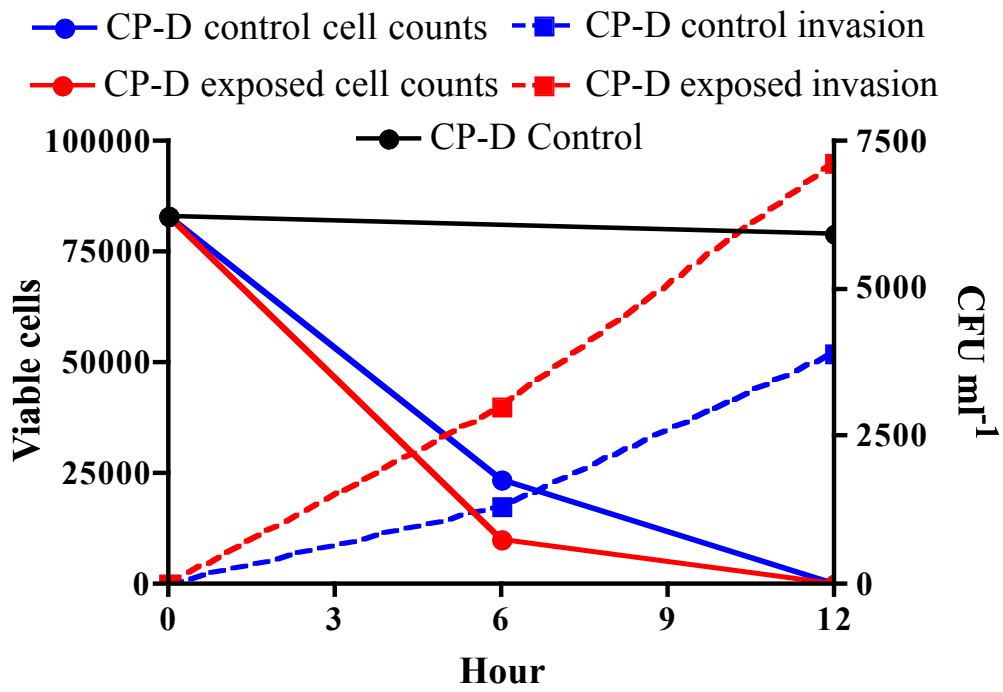


Fig. 5.10: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with a biofilm community removed from the second oesophageal chemostat with the cell line, CP-D. Control, uninfected (black).*

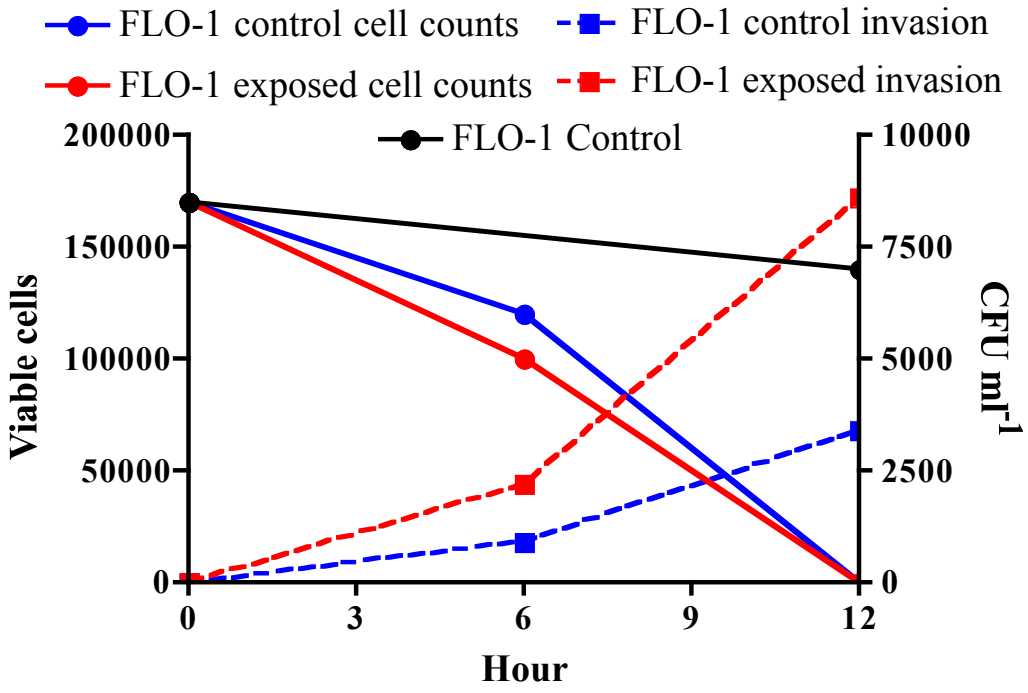


Fig. 5.11: *Counts of viable cells and bacterial CFU ml^{-1} over 24 hours of co-culture with a biofilm community removed from the second oesophageal chemostat with the cell line, FLO-1. Control, uninfected (black).*

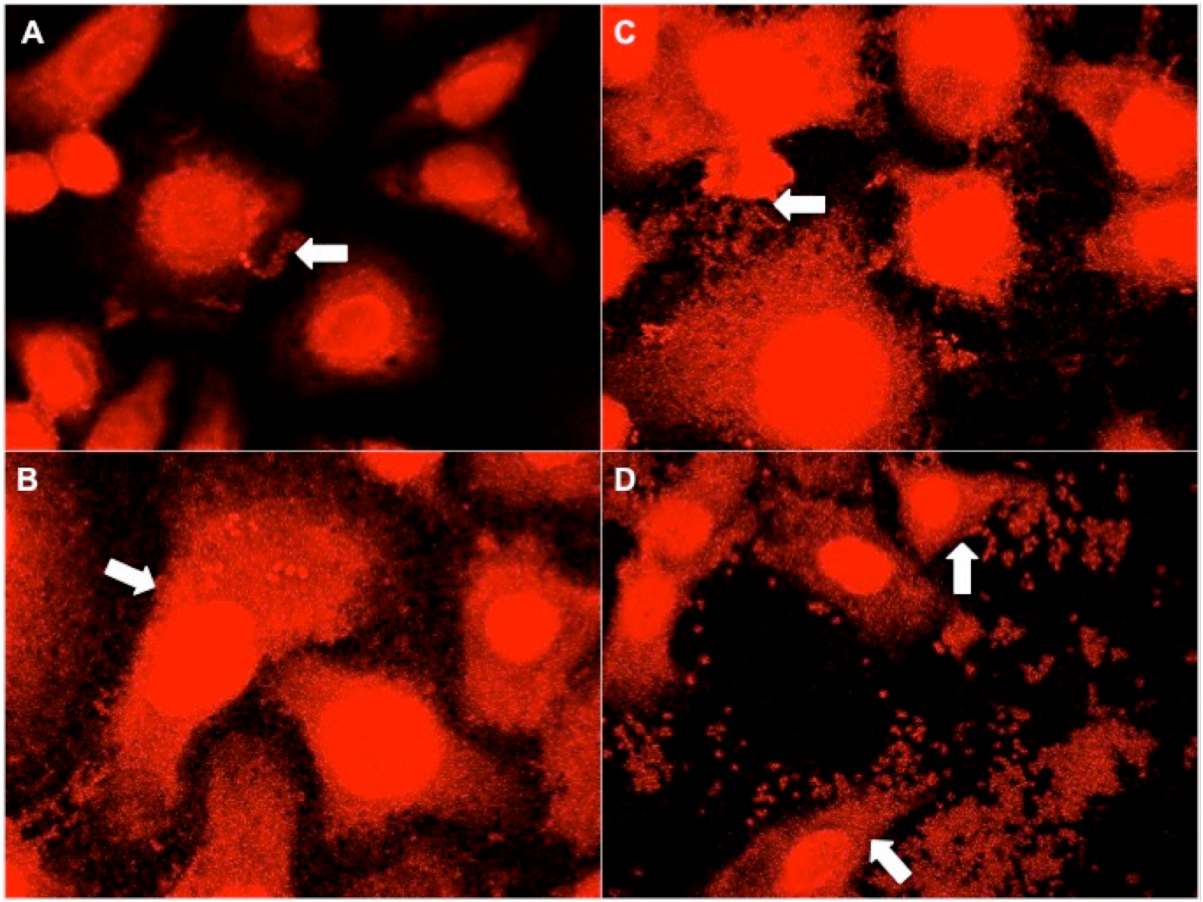


Fig. 5.12: *Images of exposed chemostat biofilms co-cultured with FLO-1 and OE21 oesophageal cells over a 24 hour period. A, FLO-1 cells after 3 hours, with cocci aggregates seen attaching to cells. B, OE21 cells after 3 hours, with bacteria seen attaching to cells and disrupting membranes. C, FLO-1 cells after 12 hours, possible bacterial cells attached, with cell material breaking down. D, OE21 cells after 12 hours, possible large infiltrates of bacteria surrounding cells, or dead cell material. Eukaryotic and bacterial cells stained with propidium iodide. All images, original magnification x 60.*

5.3.3 Western blot and immunohistochemistry analysis

Duplicate cell samples were taken from co-culture experiments for western blot analysis of p53, p21 and COX-2. Due to the small number of cells in each sample, these duplicates were pooled for improved analysis. Firstly, β-actin antibodies were utilized to confirm presence of cells; results are shown in Fig. 5.13. These blots confirm that after 12 hours of exposure to biofilms from the second chemostat run, all oesophageal cells were dead (Fig. 5.13, lanes J and M), while no β-actin was detected from CP-D cells exposed to chemostat biofilms (data not shown). Image J software allowed quantitative analysis of each band for COX-2 (Figs. 5.14 and 5.15) and p53 (Figs. 5.16 and 5.17) blots, with each cell line and environment grouped together (Figs. 5.15 and 5.17, columns 1-13).

Immunohistochemical analysis of Ki-67 did not yield any results, with changes in protein expression only seen between different cell lines, with no change after co-culture (data not show).

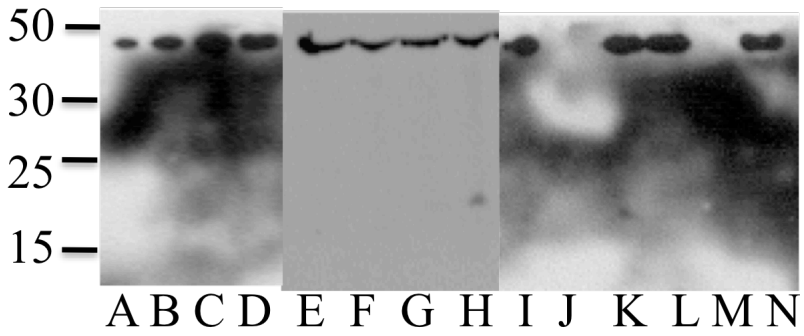


Fig. 5.13: *Blots of β-actin (42 kDa) for cells co-cultured with Campylobacter concisus and chemostat biofilms. A and C are FLO-1 with NWBO1, 24 hours and control, respectively. B and D are CP-B with NWBO1, 24 hours and control, respectively. E and F are CP-A with NWBO1, 24 hours and control, respectively. G and H are CP-D with NWBO1, 24 hours and control, respectively. I, J, K, L and M are FLO-1 cells: 6 and 12 hour control, negative, and 6 and 12 hour exposed biofilms, respectively. N is a positive β-actin control.*

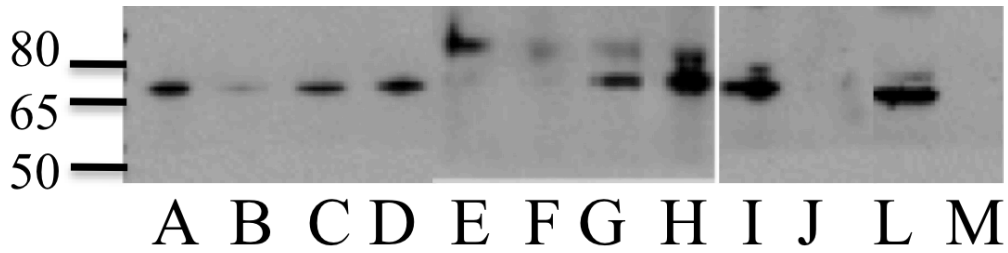


Fig. 5.14: *Blots of COX-2 (72 kDa) for cells co-cultured with Campylobacter concisus and chemostat biofilms. A and C are FLO-1 cells with NWBO1, 24 hours and control, respectively. B and D are CP-B with NWBO1, 24 hours and control, respectively. E and F are CP-A with NWBO1, 24 hours and control, respectively. G and H are CP-D with NWBO1, 24 hours and control, respectively. I, J, L, M are FLO-1 cells: 6 and 12 hour control and exposed biofilm, respectively.*

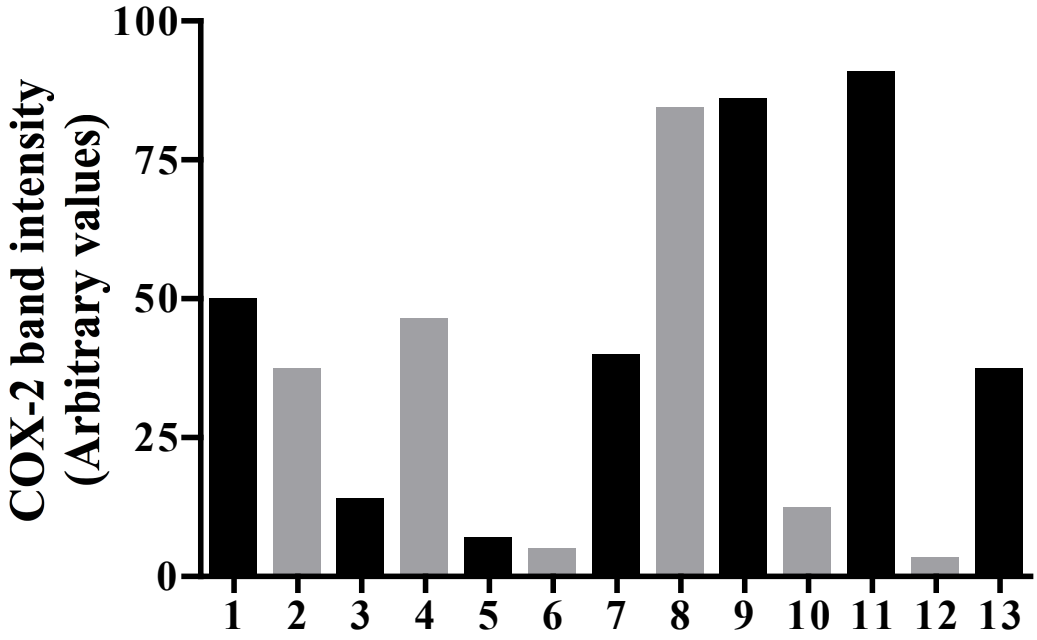


Fig. 5.15: *Graph for intensity of bands in COX-2 blots, for cells co-cultured with Campylobacter concisus and chemostat biofilms. FLO-1, CP-B, CP-A and CP-D cell lines after 24 hours of exposure to NWBO1 and a control, columns 1-8 respectively. FLO-1 cells: 6 and 12 hour control (9, 10), exposed biofilm (11, 12), and control (13).*

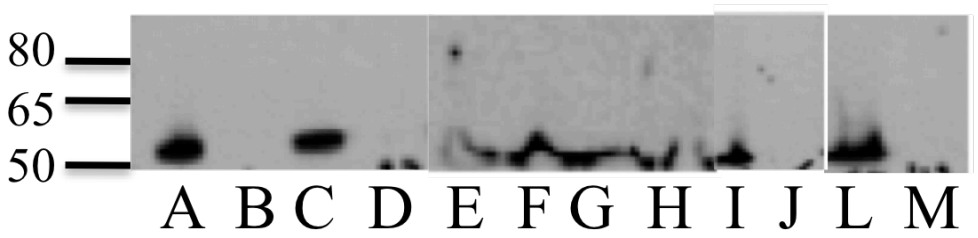


Fig. 5.16: *Blots of p53(53 kDa) for cells co-cultured with Campylobacter concisus and chemostat biofilms. See legend to Fig. 5.14 for details of samples and conditions.*

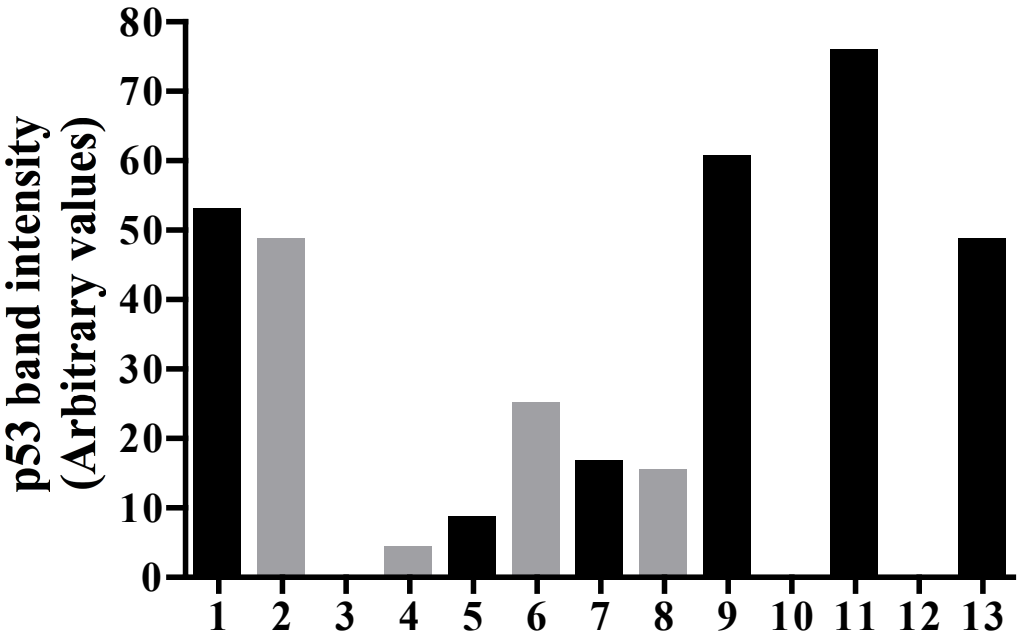


Fig. 5.17: *Graphs for intensity of bands in p53 blots, for cells co-cultured with Campylobacter concisus and chemostat biofilms. See legend to Fig. 5.15 for details of samples and conditions.*

5.4 Discussion

Work outlined in this chapter was designed to be an initial investigation of the effects of oesophageal communities on host epithelia *in vitro*. Despite time constraints and a lack of sample data, these results highlight a number of interesting observations, which, with future research, could help elucidate this Barrett's oesophagus conundrum: are specific bacteria or alterations in the whole microbiome involved in ADC development, or a consequence of changes in cellular morphology and exposure to gastric secretions?

A range of oesophageal cell lines were employed in these experiments to determine changes in individual cell type responses when in co-culture, however, every experiment was not carried out with all cell lines. If this research were pursued, then understanding the effects of bacteria on varying cell types, with a range of host and genetic response mechanisms would be invaluable. Additionally, there is a non-cancerous oesophageal cell line, HET-1A (Stoner *et al.*, 1991), which would allow full analysis of the variation in host response to infection with cell transformation.

There were dramatic variations in response to both campylobacter and multi-species biofilms between eukaryotic cell types. When exposed to *C. concisus* alone, CP-D required little invasion compared with FLO-1 to allow apoptosis, although survival was 63% *vs.* 14% at 24 hours respectively. Conversely, when co-cultured with a multi-species biofilm, invasion level at 6 hours was similar for both, with a cell survival of 28% in CP-D compared with 70% in FLO-1. This suggests resistance to invasion and an improved host response to damage by the highly dysplastic cell line, CP-D, when

exposed to *Campylobacter concisus.* Biofilm assays suggest that this cell type could not survive invasion of multiple species leading to rapid cell death. The composition of this biofilm is unknown. However, if campylobacter species were present, in combination with other potential pathogens such as fusobacteria and capnocytophaga, virulence potential could be amplified giving credence to the polymicrobial disease theory. Campylobacter exerted varied effects on all BO and HGD cell lines (CP-A, -B and –D). These three Barrett's cell forms have distinct karyotypes, with a number of abnormalities (Palanca-Wessels *et al.*, 2003). CP-A was isolated from a BO patient, with no HGD, LOH or p53 mutation, in contrast to CP-B and CP-D. When exposed to campylobacter, CP-A had reduced expression of p53, which, dependant on PCNA status (proliferating cell nuclear antigen) could lead to continued replication of DNA, cell proliferation, and consequently, ADC.

PCNA is a protein involved in DNA replication and repair. In response to DNA damage, p53 interacts with a number of proteins, such as MDM2, to determine cell fate. With increased expression of p53, its associated binding to PCNA results in DNA repair. However, if PCNA has a low abundance, is non-functional or inactivated, apoptosis occurs. Therefore, the PCNA status of these cell lines may account for the differences in response, where an increased p53 production in the presence of PCNA can result in continued survival of the cell due to DNA repair (Paunesku *et al.*, 2001). It is possible that PCNA is present in higher quantities in FLO-1, while in the Barrett's cell lines, both PCNA and p53 have a low abundance or are mutated. Hritz *et al.* (2009) measured the expression of both p53 and PCNA in BO, revealing a progressive and correlated

increase with malignant transformation. Hence, future studies may gain further understanding of the effects of bacterial stress on oesophageal cells through measurement of PCNA abundance using immunohistochemistry. The progression of chronic gastritis through the metaplasia-dysplasia sequence to cancer is associated with an accumulation of mutant p53 protein (Shiao *et al.*, 1994). A study by Kodama *et al.* (2007) found that mutant p53 is increasingly expressed in the presence of *H. pylori* during gastric carcinogenesis, with a reduced abundance on eradication. In the present study, the antibody, DO1, was utilised to measure wild-type p53, however, future studies would benefit from assessment of mutant p53 presence after co-culture with oesophageal bacteria.

This study would benefit from further work, such as the repetition of experiments to confirm the data. Further analysis of both immunological and genetic responses to oesophageal communities, in particular, NF-kappa B, PCNA, Bcl-2, and mutant p53, may reveal the importance of biofilm composition on disease progression. Data suggests that oesophageal cells have varying susceptibilities to bacteria, with cell type and genetics influencing the microbiomes invasive and aetiologic abilities. Additionally, results from co-culture of chemostat biofilms imply that bacteria complexed into a biofilm have increased pathogenicity, especially after exposure to bile acid, related to increased p53 and COX-2 expression. Further research into the virulence potential of oesophageal bacteria, specifically campylobacters, both with and without bile acid stress, may provide insight into the varied cellular responses involved in malignant transformation.

Chapter 6

General Discussion

6.1 Introduction

Oesophageal adenocarcinoma is becoming increasingly diagnosed, being the ninth most prevalent cancer in the UK, with a gender preference to males. Due to its high mortality rate, this disease is now the sixth most common cause of cancer death in the UK (CRUK, 2010). Human intestinal microorganisms play an essential role in the maintenance of health, with their ecology and metabolism being influenced by changes in host environmental conditions. Bacteria are associated with a number of cancers (Section 1.4.1), with *H. pylori* involvement in gastric cancer providing a paradigm for comparisons with oesophageal adenocarcinoma. This study demonstrated an association between changes in oesophageal microbiota composition and the presence of campylobacters, with progression of oesophageal disease. However, causality could not be elucidated, therefore, the "chicken or egg" question remains: does an individual's microbiota initiate disease, or does reflux result in microbial shifts, possibly leading to disease maintenance, and progression to malignancy?

Work presented in this thesis details cultural and molecular analyses of a large cohort of patients with GORD, BO and ADC, comparing their microbial profiles with those of normal healthy subjects. Development of a model oesophageal community provided information on biofilm composition and the effects of bile and acid refluxate on both the microbiota and its associated oesophageal epithelial response. Improved understanding of the oesophageal microbiome and its involvement in ADC development will allow better treatment regimes to be developed, with a possible role for probiotics and prebiotics to beneficially alter community structure, and therefore, gut health.

6.2 Oesophageal communities in the progression to adenocarcinoma

Clinical studies (Chapters 2 and 3) indicate that as the oesophagus becomes increasingly damaged by reflux, mucosal populations are affected. As disease progresses down the metaplastic-dysplastic sequence from GORD, the microbiota becomes increasingly varied and Gram negative. The phyla and genera identified in GORD and BO match those from previous studies using PCR clone libraries (Pei *et al.*, 2004, 2005; Yang *et al.*, 2009), with genera representing Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria and Bacteroidetes. Advancing from these initial studies into Barrett's oesophagus, tissues were collected from patients with adenocarcinoma. Traditional culture identified 23 genera and 73 species in ADC compared with 19 genera and 56 species in controls. Adenocarcinoma patients had a continually more diverse population represented by an increase in Gram negative species such as campylobacters and fusobacteria, and Gram positive organisms such as *Actinomyces, Lactobacillus* and *Peptostreptococcus*. Molecular studies using real-time PCR permitted a greater number of samples to be analysed, however, only a selection of bacterial assays were designed. Data from this part of the study found a significant reduction in fusobacteria, bifidobacteria, veillonella and *H. pylori*.

Results from Macfarlane *et al.* (2007) found *C. concisus* in 57% of BO patients, in this study 75%, 50% and 60% of GORD, BO and ADC patients respectively, were found to have these viable organisms (51%, 42%, and 8% respectively, using molecular techniques (Chapter 3)). Cultural analysis identified *C. concisus* as the main species found, most often in complex with other campylobacters and a range of other Gram negative species such as leptotrichias, arcobacters and fusobacteria.

Additionally, results from both analytical approaches identified diminished colonisation by fusobacteria, mostly in GORD patients. As discussed throughout this dissertation, mucosal populations and their community dynamics are essential for maintenance of health. Fusobacteria are an integral component of oral biofilms, being a bridge organism for aggregation of species such as veillonella. The theory of a whole shift in the microbiome (type I and II) in oesophageal health and disease (Yang *et al.*, 2009) fits with data presented in this study. Discrepancies exist between these two studies, most markedly for streptococci, although both identified a more varied, anaerobic and Gram negative microbial status in disease. The reduction of both streptococci and fusobacteria in GORD patients would alter the community dynamic, with opportunity for other species to colonise. *Campylobacter*, an opportunistic pathogen, is proposed in Chapter 2, to form a complex with a range of potential pathogens, which could enhance overall pathogenicity, explicated by the polymicrobial disease theory, similar to that of the oral "Red Complex" involving treponema and porphyromonas (Socransky *et al.*, 1998). Interestingly, a recent mouse model study identified a sexual dimorphism in *C. jejuni*, with increased colonisation of this organism in male mice (Strachan *et al.*, 2008). A study into the prevalence of ADC in North America, Europe and Australia since 1960, analysed all data with a unified exponential growth model, finding that rates of oesophageal ADC in males are increasing, most prominently in the UK (Bollschweiler *et al.*, 2001). Therefore, campylobacters, possibly in an aggregated complex, may by a factor in malignancy.

In diseased tissue, levels of bifidobacteria, staphylococci, bacteroides, veillonella and *H. pylori* were reduced compared with a control tissue from the same individual,

with levels of *H. pylori* being significantly lower in all disease patients compared with healthy controls. A reduction in *Bacteroidetes* has recently been associated with obesity (Wexler, 2007), which is a common predisposition to oesophageal disease. While the loss of *H. pylori* from the oesophagus in disease, and furthermore, in tumourous tissue, gives credence to the hypothesis of protection by this species (Lochhead and El-Olmar, 2008; Atherton, 2008).

6.3 *In vitro* models of the oesophageal microbiota

A two-stage continuous culture system was developed to establish populations representative of those found in the mouth, which would consequently lead to the development of diverse and unique communities similar to those of the oesophagus. Initial microbiological results indicated that this attempt was successful with two distinct communities generated, supporting the theory of oesophageal colonisation from the oral cavity. After exposure of these oesophageal model biofilms to bile acid cocktail, a shift in composition was identified, with a reduced total CFU composed of a greater variety of species. Additionally, as seen clinically in oesophageal disease, these populations had a greater proportion of Gram negative species. When exposed to oesophageal cell lines, these stressed communities resulted in a more rapid invasion, and consequently, cell death, compared with those not exposed to refluxate. This observation is most likely due to an increased haemolytic activity, with exposed biofilms expressing higher levels of haemolysin. Furthermore, these stressed communities had increased expression of mucinolytic enzymes, which, if representative of the situation *in vivo* would lead to increased invasion potential, inflammation and possibly tumour development, through recruitment of cytokines

and upregulation of genes for COX-2 protein (Macarthur *et al.*, 2004), as seen with FLO-1 cells (Figs. 5.15 and 5.17).

6.4 Main conclusions

There is growing evidence that prior and chronic bacterial infections are linked to tumour formation, although the molecular mechanisms involved remain to be elucidated. These results imply that as cell morphology changes the colonisation potential of bacteria is altered, with a resulting shift in the microbiota, yet this is only one explanation. It is also possible that an individual's microbiome determines their cellular response to refluxate, with opportunistic pathogens increasing their virulence potential under adverse conditions, leading to chronic inflammation and tumour formation.

There are a number of possible mechanisms attributable to bacterial involvement, the first being inflammation due to induction of the immune response. While interference with the cell cycle and its associated signalling pathways, such as TNF-α, Bcl-2 and COX-2, would also result in continued tumour survival and invasion (Lax and Thomas, 2002). Finally, bacteria may metabolise potentially carcinogenic compounds. Oral squamous cell carcinoma is associated with alcohol consumption, as it is proposed that bacterial metabolism of ethanol to acetaldehyde results in DNA damage and mutagenesis (Hooper *et al.*, 2006). In the case of oesophageal adenocarcinoma, the presence of gastric acid in the distal oesophagus may amplify the production of NO from dietary nitrate by mucosal populations, specifically the nitrate-reducing *Campylobacter* species. Additionally, presence of the saccharolytic bacteria, lactobacillus, was increased in disease, this species can produce SCFA from

carbohydrates, further increasing the environmental acidity. This species was isolated from oral tumour sites (Hooper *et al.*, 2006), and may also be involved in oesophageal ADC, either due to epithelial damage, or modification of the mucosal populations due to selection of acid tolerant species.

It was hypothesised that in ADC, microbial composition would vary, becoming more diverse and Gram negative, with a possible key role for campylobacters. Data suggests that this is correct, however, the role of campylobacter cannot be fully understood without further mechanistic studies. It seems most likely that reflux disease due to host characteristics disrupts the microbiome composition, rather than individual mucosal populations initiating disease, with further exacerbation by refluxate. As bile and acid are exposed to the distal oesophagus, mucosal populations may be affected, with only tolerant species surviving. Additionally, opportunistic pathogens may thrive, with bile salts enhancing the expression of virulence proteins in these organisms (Malik-Kale *et al*., 2008), resulting in induction of the immune response, inflammation and malignant transformation. However, the other possibility of bacterial initiation should not be discounted without further research into the associations of *H. pylori* with the commensal oesophageal community. Additionally, research is essential to better understand the prevalence and virulence status of *C. concisus*, the associated effects of refluxate exposure, and inter-individual variations in the host microbiome.

6.5 Future work

This study represents a preliminary investigation of bacterial colonisation in the malignant oesophagus, with much still to be learned about the host microbiota and its mechanisms in disease. If further research was pursued, there are a number of avenues that could be taken:

1. To further understand the effect of changes in cell morphology on biofilm composition, further tissue samples need to be collected from GORD, BO and ADC patients, with matched control squamous tissues. This would allow determination of the changes in microbiota composition, and also the associated immune and oncogenic response with malignancy.

2. Understanding of bacterial involvement in disease initiation or maintenance would benefit from a long-term study with "at risk" patients from the start of the disease sequence. Removal of oesophageal tissues periodically, at both the diseased, and a control site in each patient, for investigation of the microbiota, may reveal a single or complexed aetiologic agent. These investigations would provide full information on the shifts in microbial composition over time, in both patients who do, and do not, progress to adenocarcinoma. A follow on clinical trial with probiotics, or a synbiotic containing *Bifidobacterium*, in conjunction with microbial, histological and genetic analysis may provide insight into mechanisms of disease and possible treatments.

3. Full genetic analysis of campylobacter species, and other potential aetiologic agents, would provide insight into the presence of pathogenicity islands, and

other sources of virulence genes. Continuous and batch cultures of these organisms with various bile salts and acids, before repeated genetic sequencing, may highlight the stress response in these organisms, with possible upregulation of haemolysins, toxins and flagellins, representative of virulence potential.

4. Results from cell culture experiments (Chapter 5), suggest that bacteria play an important role in oesophageal disease. Therefore, a full investigation with a range of oesophageal cells, both immortalised and primary, with individual agents and a full biofilm, may shed light on these important interactions. Additionally, exposure of these bacteria to refluxate would provide a better understanding of host physiology and microbiome ecology in reflux disease. Finally, these co-cultured cells could undergo genetic and proteomic analysis to measure effects of single and multiple species, before and after stress, and the effects of treatment with commensal and probiotic bacteria. Measurement of a range of oncogenes, immune and stress response proteins, cell-cycle regulators and transcription factors involved in progression of GORD to ADC, should be investigated.

5. If, with extensive research, it is proven that bacteria have an important role to play in oesophageal pathogenesis, animal models could be developed. It would be of great interest to infect these models with oesophageal cultures from patients with GORD, BO and ADC, to observe whether inflammation and tumourigenesis could be initiated.

Bibliography

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 2005;43(11):5721-5732.

Adler RH. The lower esophagus lined by columnar epithelium: its association with hiatal hernia, ulcer, stricture and tumour. *J Thorac Cardiovasc Surg* 1963;45:13-32.

Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004;4:499-511.

Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-680.

Allen KJ, Griffiths MW. Effect of environmental and chemotactic stimuli on the activity of the *Campylobacter jejuni* flaA sigma (28) promoter. *FEMS Microbiol Lett* 2001;205(1):43-48.

Allison CN, Coleman PL, Jones PL, Hughes C. Ability of *P. mirabilis* to invade human urothelial cells is couple to motility and swarming differentiation. *Infect Immun* 1992;60:4740-4746.

Allison PR. Reflux esophagitis, sliding hiatal hernia, and the anatomy of repair. *Surg Gynecol Obstet* 1951;92:419-431.

Allison PR, Johnstone AS. The oesophagus lined with gastric mucous membrane. *Thorax* 1953;8:87-101.

Allweiss BJ, Dostal K, Carey E, Edwards TF, Freter R. The role of chemotaxis in the ecology of bacterial pathogens of mucosal surfaces. *Nature* 1977;266:448-450.

Anderson LA, Murphy SJ, Johnston BT, Watson RGP, Ferguson HR *et al.* Relationship between *Helicobacter pylori* infection and gastric atrophy and the stages of the oesophageal inflammation, metaplasia, adenocarcinoma sequence: results from the FINBAR case control study. *Gut* 2008;57:734-739.

Aries V, Crowther JS, Drasar BS, Hill MJ. Degradation of bile salts by human intestinal bacteria. *Gut* 1969;10(7):575-576.

Ashimoto A, Chen C, Kakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;11:266-273.

Atherton J. Does *Helicobacter pylori* protect against oesophageal adenocarcinoma, and if so, how? *Clin Med* 2008;8:420-422.

Avila M, Ojcius DM, Yilmaz O. The oral microbiota: living with a permanent guest. *DNA Cell Biol* 2009;28(8):405-411.

Barak N, Ehrenpreis ED, Harrison JR, Sitrin MD. Gastro-oesophageal reflux disease in obesity: pathophysiological and therapeutic considerations. *Obes Rev* 2002;3:9-15.

Bardy SL, Ng SY, Jarrell KF. Prokaryotic motility structures. *Microbiol* 2003;149:295-304.

Barham CP, Jones RL, Biddlestone LR, Hardwick RH, Shepherd NA *et al.* Photothermal laser ablation of Barrett's oesophagus: endoscopic and histological evidence of squamous re-epithelialisation. *Gut* 1997;41:281-284.

Barrett NR. Chronic peptic ulcer of the oesophagus and oesophagitis. *Br J Surg* 1950;35:175-182.

Barrett NR. The lower esophagus lined by columnar epithelium. *Surgery* 1957;41:881–894.

Beerens H. An elective and selective isolation medium for *Bifidobacterium* spp. *Lett App Microbiol* 1990;11:155-157.

Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev* 2005;29:625-651.

Bennet JR. pH measurement in the oesophagus. *Baillieres Clin Gastroenterol* 1987;1(4):747-767.

Bernhard AE, Field KG. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S Ribosomal DNA genetic markers from fecal anaerobes. *Appl Env Microbiol* 2000;66(4):1587-1594.

Berstad A. Relationship of overweight to hiatus hernia and reflux oesophagitis. *Scand J Gastroenterol* 1988;23:427-432.

Binato M, Gurski RR, Fagundes RB, Meurer L, Edelweiss MI. P53 and Ki-67 overexpression in gastroesophageal reflux disease – Barrett's esophagus and adenocarcinoma sequence. *Dis Esophagus* 2009;22(7):588-595.

Binshtok AM, Wang H, Zimmermann K, Amaya F, Vardeh D *et al*. Nociceptors are interleukin-1beta sensors. *J Neurosci* 2008;28(52):14062-14073.

Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986;321:209-213.

Blot WJ, Devesa SS, Kneller RW, Fraumeni JF Jr. Rising incidence of adenocarcinoma of the oesophagus and gastric cardia. *JAMA* 1991;265:1287-1289.

Boeuf P, Vigan-Womas I, Jublot D, Loizon S, Barale JC *et al*. CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. *BMC Immunology* 2005;6(1):5.

Bollschweiler E, Wolfgarten E, Gutschow C, Holscher AH. Demographic variations in the rising incidence of esophageal adenocarcinoma in white males. *Cancer* 2001;92:549– 555.

Borrmann E, Berndt A, Hanel I, Kohler H. *Campylobacter*-induced interleukin-8 responses in human epithelial cells and primary intestinal chick cells. *Vet Microbiol* 2007;124:115-124.

Bosher LH, Taylor FH. Heterotopic gastic mucosa in the esophagus with ulceration and stricture formation. *J Thorac Surg* 1951;21:306-312.

Bourke B, Chan VL, Sherman P. *Campylobacter upsaliensis*: waiting in the wings. *Clin Microbiol Rev* 1998;11:440–449.

Bowden GHW, Hamilton IR. Survival of oral bacteria. *Crit Rev Oral Biol Med* 1998;9:54-85.

Bradshaw DJ, Marsh PD, Allison C, Schilling KM. Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms. *Microbiol* 1996a;142:623-629.

Bradshaw DJ, Marsh PD, Schilling KM, Cummins D. A modified chemostat system to study the ecology of oral biofilms. *J Appl Bacteriol* 1996b;80(2):124-130.

Bradshaw DJ, Marsh PD, Watson GK, Allison C. Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. *Infect Immun* 1998;66(10):4729-4732.

Bradshaw DJ, McKee AS, Marsh PD. Effects of carbohydrate pulses and pH on population shifts within the oral microbial communities *in vitro*. *J Dent Res* 1989;68(9):1298-1302.

Bry L, Falk PG, Midtvedt T, Gordon JI. A model of host-microbial interactions in an open mammalian ecosystem. *Science* 1996;273:1380-1383.

Burnett T, Pung A, Bertram JS, Cooney RV. The role of nitric oxide in neoplastic transformation of C3H 10T1/2 embryonic fibroblasts. *Carcinogenesis* 2000;21(11):1989-1995.

Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR. The chemistry of DNA damage from nitric oxide and peroyxnitrite. *Mut Res* 1999;424:37-49.

Cameron AJ, Lomboy CT. Barrett's esophagus: age, prevalence, and extent of columnar epithelium. *Gastroenterology* 1992;103(4):1241-1245.

Cancer Research UK (CRUK, 2010) Statistical Information Team. [cited July 2010]; Available from: http://info.cancerresearchuk.org/cancerstats/types/oesophagus.

Cannon WB. Conditions affecting the activities of the cardia. In: Arnold E, ed. *The Mechanical Factors of Digestion.* New York: Longmans, Green and Co., 1911:32-34.

Carrie A. Adenocarcinoma of the upper end of the oesophagus arising from ectopic gastric epithelium. *Br J Surg* 1950;37:474.

Catalano F, Terminella C, Grillo C, Biondi S, Zappala M *et al*. Prevalence of oesophagitis in patients with persistent upper respiratory symptoms. *J Laryngol Otol* 2004;118(11):857-861.

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E *et al.* Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* 2001;12(2):323-337.

Chan AO, Peng JZ, Lam SK, Lai KC, Yuen MP *et al.* Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter methylation. *Gut* 2006;55:463-468.

Code CF, Fyke FE, Schlegel JF. The gastroesophageal sphincter in healthy human beings. *Gastroenterologia* 1956;86:135-150.

Collado MC, Gonzalez A, Gonzalez R, Hernandez M, Ferrus MA *et al*. Antimicrobial peptides are among the antagonistic metabolites produced by *Bifidobacterium* against *Helicobacter pylori*. *Int J Antimicrob Agents* 2005;25:385-391.

Conio M, Cameron AJ, Romero Y, Branch C, Schleck C *et al.* Secular trends in the epidemiology and outcome of Barrett's esophagus in Olmsted County, Minnesota. *Gut* 2001;48:304-309.

Corey KE, Schmitz SM, Shaheen NJ. Does a surgical anti-reflux procedure decrease the incidence of esophageal adenocarcinoma in Barrett's esophagus? A meta-analysis. *Am J Gastroenterol* 2003;98:2390-2394.

Corley DA, Kubo A, Levin TR, Block G, Habel L *et al. Helicobacter pylori* infection and the risk of Barrett's oesophagus: a community-based study. *Gut* 2008;57:727-733.

Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D *et al*. Biofilms, the customized microniche. *J Bacteriol* 1994;176(8):2137-2142.

Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 2000;64(4):847-867.

Davies HA, Jones DB, Rhodes J, Newcombe RG. Angina-like esophageal pain: differentiation from cardiac pain by history. *J Clin Gastroenterol* 1985;7:477-481.

Day WA Jr, Sajecki JL, Pitts TM, Joens LA. Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun* 2000;68(11):6337-6345.

Dean DA, Burchard KW. Surgical perspective on invasive *Candida* infections. *World J Surg* 1998;22(2):127-134.

de Man, JD, Rogosa M, Sharpe ME. A medium for the cultivation of *Lactobacilli*. *J Appl Bact* 1960;23:130-135.

de Martel C, Llosa AE, Farr SM, Friedman DG, Vogelman JH. *Helicobacter pylori* infection and the risk of development of esophageal adenocarcinoma. *J Infect Dis* 2005;191:761-767.

Devesa SS, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998;83:2049-2053.

Diakowska D, Markocka-Maczka K, Grabowski K, Lewandowski A. Serum interleukin-12 and interleukin-18 levels in patients with oesophageal squamous cell carcinoma. *Exp Oncol* 2006;28:319-322.

Dibdin GH, Shellis RP, Wilson CM. An apparatus for the continuous culture of microorganisms on solid surfaces with special reference to dental plaque. *J Appl Bacteriol* 1976;40:261-268.

Dickman R, Green C, Chey WD, Jones MP, Eisen GM *et al*. Clinical predictors of Barrett's esophagus length. *Gastrointest Endosc* 2005;62(5):675-681.

Dicksved J, Lindberg M, Rosenquist M, Enroth H, Jansson JK *et al*. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *J Med Microbiol* 2009;58:509-516.

Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evolution of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci* 2005;113:14-19.

Doel JJ, Hector MP, Amirtham CV, Al-Anzan LA, Benjamin N *et al*. Protective effect of salivary nitrate and microbial nitrate reductase activity against caries. *Eur J Oral Sci* 2004;112:424-428.

Dorrell N, Martino MC, Stabler RA, Ward SJ, Zhang ZW *et al.* Characterisation of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa. *Gastroenterology* 1999;117:1098-1104.

D'Urzo A, Jugovic P. Chronic cough. Three most common causes. *Can Fam Physician* 2002;48:1311-1316.

Dvorak K, Chavarria M, Payne CM, Ramsey L, Crowley-Weber C *et al.* Activation of the Interleukin-6/STAT3 antiapoptotic pathway in esophageal cells by bile acids and low pH: relevance to Barrett's esophagus. *Clin Can Res* 2007;13(18):5305-5313.

Dvorakova K, Payne CM, Ramsey L, Holubec H, Sampliner R *et al.* Increased expression and secretion of Interleukin-6 in patients with Barrett's Esophagus. *Clin Cancer Res* 2004;10:2020-2028.

Ell C, May A, Gossner L, Pech O, Gunter E, *et al*. Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's esophagus. *Gastroenterology* 2000;118(4):670-677.

El-Serag HB, Aguirre T, Kuebeler M, Sampliner RE. The length of newly diagnosed Barrett's oesophagus and prior use of acid suppressive therapy. *Aliment Pharmacol Ther* 2004;19(12):1255-1260.

El-Serag HB, Tran T, Richardson P, Ergun G. Anthropometric correlates of intragastric pressure. *Scand J Gastroenterol* 2006;41(8):887-891.

Engberg J, Bang DD, Aabenhus FM, Fussing V, Gerner-smidt P. *Campylobacter concisus*: an evaluation of certain phenotypic and genotypic characteristics. *Clin Microbiol Infect* 2005;11:288-295.

Eslick GD, Jones MP, Talley NJ. Non-cardiac chest pain: prevalence, risk factors, impact and consulting – a population-based study. *Aliment Pharmacol Ther* 2003;17:1115-1124.

Falk GW. Barrett's esophagus. *Gastroenterology* 2002;122:1569-1591.

Fauchere JL, Rosenau A, Veron M, Moyen EN, Richard S *et al*. Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human feces. *Infect Immun* 1986;54:283-287.

Fein M, Ritter MP, De Meester TR, Oberg S, Peters JH *et al*. Role of the lower esophageal sphincter and hiatal hernia in the pathogenesis of gastroesophageal reflux disease. *J Gastrointest Surg* 1999;3(4):405-410.

Feith M, Stein HJ, Mueller J, Siewert JR. Malignant degeneration of Barrett's esophagus: the role of the Ki-67 proliferation fraction, expression of E-cadherin and p53. *Dis Esophagus* 2004;17:322-327.

Filoche S, Wong L, Sissons CH. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res* 2010;89(1):8-18.

Finlay IG, Wright PA, Menzies T, McArdle CS. Microbial flora in carcinoma of the oesophagus. *Thorax* 1982;37:181-184.

Fitzgerald RC, Abdalla S, Onwuegbusi BA, Sirieix P, Saeed IT *et al.* Inflammatory gradient in Barrett's oesophagus: implications for disease complications. *Gut* 2002a;51(3):316-322.

Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliot M, Saeed IT, Burnham WR *et al*. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. *Gut* 2002b;50:451-459.

Fitzgerald RC, Saeed IT, Khoo D, Farthing MH, Burnham WR. Rigorous surveillance protocol increases detection of curable cancers associated with Barrett's esophagus. *Dig Dis Sci* 2001;46:1892-1898.

Flegal KM, Carroll MD, Kuczmarski RJ, Johnson CL. Overweight and obesity in the United States: prevalence and trends, 1960-1994. *Int J Obes Relat Metab Disord* 1998;22(1):39-47.

Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* 2002;288(14):1723-1727.

Friis LM, Keelan M, Taylor DE. *Campylobacter jejuni* drives MyD88-independent Interleukin-6 secretion via Toll-like receptor 2. *Infect Immun* 2009;77(4):1553-1560.

Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV *et al*. Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005a;54(2):242-249.

Furrie E, Macfarlane S, Thomson G, Macfarlane GT. Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. *Immunology* 2005b;115:565-574.

The Gallup Organization. Gallup Survey on Heartburn Across America. Princeton, NJ, The Gallup Organization Inc., 1988.

Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J Appl Bacteriol* 1994;77:412-420.

Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC *et al. Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci USA* 2001;98:13844-13849.

Golub EE, Thaler M, Davis C, Malamud D. Bacterial aggregating activity in human saliva: a simultaneous determination of free and bound cells. *Infect Immun* 1979;26(3):1028-1034.

Grant CCR, Michael EK, Witold CJR, Lucy ST. Role of flagella in adherence, internalisation and translocation of *Campylobacter jejuni* in non polarized and polarized epithelial cell cultures. *Infect Immun* 1993;61:1764-1771.

Guerry P. *Campylobacter* flagella: not just for motility. *Trends Microbiol* 2007;15(10):456-461.

Haggitt RC, Tryzellar J, Ellis FH, Colcher H. Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. *Am J Clin Pathol* 1978;70:1-5.

Hampel H, Abraham NS, El-serag HB. Meta-analysis: obesity and the risk of GORD and its complications. *Ann Intern Med* 2005;143(3):199-211.

Hannu T, Mattila L, Rautelin H, Pelkonen P, Lahdenne P *et al*. *Campylobacter*-triggered reactive arthritis: a population-based study. *Rheumatology* 2002;41(3):312-318.

Hawe A, Payne WS, Welland LH. Adenocarcinoma in the columnar epithelial lined lower (Barrett) esophagus. *Thorax* 1973;28:511-514.

Hayward J. The lower end of the oeosphagus. *Thorax* 1961;16:36-41.

Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD *et al*. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl Environ Microbiol*. 2002;68:114-123.

Hida N, Shimoyamam T Jr, Neville P, Dixon MF, Axon AT *et al.* Increased expression of IL-10 and IL-12 (p40) mRNA in *Helicobacter pylori* infected gastric mucosa: relation to bacterial *cag* status and peptic ulceration. *J Clin Pathol* 1999;52:658-664.

Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (NY)* 1993;11(9):1026-1030.

Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. *Science* 2001;292:1115-1118.

Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 2002;22:283-307.

Hooper SJ, Crean SJ, Lewis MAO, Spratt DA, Wade WG *et al.* Viable bacteria present within oral squamous cell carcinoma tissue. *J Clin Microbiol* 2006;44(5):1719-1725.

Hopkins MJ, Sharp R, Macfarlane GT. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. *Gut* 2001;48:198-205.

Hritz I, Gyorffy H, Molnar B, Lakatos G, Sipos F *et al.* Increased p53 expression in the malignant transformation of Barrett's esophagus is accompanied by an upward shift of the proliferative compartment. *Pathol Oncol Res* 2009;15(2):183-192.

Hu L, Bray MD, Osorio M, Kopecko DJ. *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infect Immun* 2006;74(5):2697-2705.

Huebner RJ, Todaro GJ. Oncogenes of RNA tumor viruses as determinants of cancer. *Proc Natl Acad Sci USA* 1969;64(3):1087-1094.

Hugdahl MB, Beery JT, Doyle MP. Chemotactic behavior of *Campylobacter jejuni*. *Infect Immun* 1988;56(6):1560-1566.

Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer* 2007;121(11):2373-2380.

Ihara H, Miura T, Kato T, Ishihara K, Nakagawa T *et al*. Detection of *Campylobacter rectus* in periodontitis sites by monoclonal antibodies. *J Periodontal Res* 2003;38(1):64-72.

Iijima K, Henry E, Moriya A, Wirz A, Kelman W *et al*. Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastro-oesophageal junction. *Gastroenterology* 2002;122:1248-1257.

Irani S, Parkman HP, Thomas R, Krevsky B, Fisher RS *et al.* Increased Barrett's esophagus for the decade between 1991 and 2000 at a single university medical center. *Dig Dis Sci* 2005;50:2141-2146.

Isomoto H, Inoue K, Kohno S. Interleukin-8 levels in esophageal mucosa and long-term clinical outcome of patients with reflux esophagitis. *Scand J Gastroenterol* 2007;43:410-411.

Isomoto H, Wang A, Mizuta Y, Akazawa Y, Ohba K *et al.* Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. *Am J Gastroenterol* 2003;98:551-556.

Istivan TS, Coloe PJ, Fry BN, Ward P, Smith SC. Characterisation of a haemolytic phospholipase A(2) activity in clinical isolates of *Campylobacter concisus*. *J Med Microbiol* 2004;53:483-493.

Istivan TS, Smith SC, Fry BN, Coloe PJ. Characterisation of *Campylobacter concisus* hemolysins. *FEMS Immunol Med Microbiol* 2008;54(2):224-235.

Jailwala JA, Shaker R. Oral and pharyngeal complications of gastroesophageal reflux disease. globus, dental erosions, and chronic sinusitis. *J Clin Gastroenterol* 2000;30(3 Suppl.):S35-S38.

Jain SK, Jain S, Jain M, Yaduvanshi A. Esophageal tuberculosis: is it so rare? Report of 12 cases and review of the literature. *Am J Gastroenterol* 2002;97(2):287-291.

Jin Z, Cheng Y, Gu W, Zheng Y, Sato F *et al.* A multicentre, double-blinded validation study of methylation biomarkers for progression prediction in Barrett's esophagus. *Cancer Res* 2009;69(10):4112-4115.

Johnson WM, Lior H. Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. *FEMS Microbiol Lett* 1987;43(1):19-23.

Johnson WM, Lior H. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microb Pathog* 1988;4(2):115-126.

Johnston MH. Technology insight: ablative techniques for Barrett's esophagus – current and emerging trends. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:323-330.

Jones AD, Bacon KD, Jobe BA, Sheppard BC, Deveney CW *et al*. *Helicobacter pylori* induces apoptosis in Barrett's-derived esophageal adenocarcinoma cells. *J Gastrointest Surg* 2003;7(1):68-76.

Jung HC, Eckmann L, Yang SK, Panja A, Fierer J *et al*. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* 1995;95:55-65.

Kang JH, Lee S, Kim JS, Jung HY. Profile of aberrant CpG island methylation along multistep gastric carcinogenesis. *Lab Invest* 2003;83:519-526.

Katz PO, Zavala S. Proton pump inhibitors in the management of GERD. *J Gastrointest Surg* 2010;14(Suppl 1):S62-66.

Kaur BS, Khamnehei N, Iravani M, Namburu SS, Lin O *et al*. Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus. *Gastroenterology* 2002;123(1):60-67.

Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K *et al*. Natural antibiotic function of a human gastric mucin against *Helicobacter pylori* infection. *Science* 2004;305:1003–1006.

Kawasaki S, Fratamico PM, Wesley IV, Kawamoto S. Species-specific identification of Campylobacters by PCR-restriction fragment length polymorphism and PCR targeting of the gyrase B gene. *Appl Environ Microbiol* 2008;74(8):2529-2533.

Kim SW, Kim HM, Yang KM, Kim SA, Kim SK *et al. Bifidobacterium lactis* inhibits NF-kappa B in intestinal epithelial cells and prevents acute colitis and colitis-associated colon cancer in mice. *Inflamm Bowel Dis* 2010;16(9):1514-1525.

Kleppe K, Ohtsuka E, Kleppe R, Molineux I, Khorana HG. Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalalyzed by DNA polymerases. *J Mol Biol* 1971;56:341-361.

Kodama M, Murakami K, Okimoto T, Sato R, Watanabe K *et al*. Expression of mutant type-p53 products in *H. pylori*-associated chronic gastritis. *World J Gastroenterol* 2007;13(10):1541-1546.

Kok RG, de Waal A, Schut F, Welling GW, Weenk G *et al.* Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. *Appl Env Microbiol* 1996;62(10):3668-3672.

Kolenbrander PE. The genus *Veillonella*. In: Dworkin MM, Falkow S,Rosenberg E, Schleifer KH, Stackebrandt E, ed. *The Prokaryotes*. Springer New York, 2006;1022-1040.

Korczak BM, Stieber R, Emler S, Burnens AP, Frey J *et al*. Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. *Int J Syst Evol Microbiol* 2006;56:937-945.

Koyi H, Branden E, Gnarpe J, Gnarpe H, Steen B. An association between chronic infection with *Chlamydia pneumoniae* and lung cancer. A prospective 2-year study. *APMIS* 2001;109(9):572-580.

Kreth J, Merritt J, Qi F. Bacterial and host interactions of oral streptococci. *DNA Cell Biol* 2009;28(8):397-403.

Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML *et al*. New bacterial species associated with chronic periodontitis. *J Dent Res* 2003;82:338-344.

Kumar S, Kumar S, Kumar S. Infection as a risk factor for gallbladder cancer. *J Surg Oncol* 2006;93(8):633-639.

Kuwabara S. Guillain-Barre syndrome epidemiology, pathophysiology and management. *Drugs* 2004;64:597-610.

Kuwabara S, Ogawara K, Misawa S, Koga M, Mori M *et al*. Does *Campylobacter jejuni* infection elicit "demyelinating" Guillain-Barre syndrome? *Neurology* 2004;63(3):529-533.

Lao-Sirieix P, Caldas C, Fitzgerald RC. Genetic predisposition to gastro-oesophageal cancer. *Curr Opin Genet Dev* 2010;20(3):210-217.

Lau WF, Wong J, Lam KH, Ong GB. Oesophageal microbial flora in carcinoma of the oesophagus. *Aust N Z J Surg* 1981;51:52-55.

Lax AJ, Thomas W. How bacteria could cause cancer: one step at a time. *Trends Microbiol* 2002;10(6):293-299.

Lebel-Binay S, Berger A, Zinzindohoué F, Cugnenc P, Thiounn N *et al.* Interleukin-18: biological properties and clinical implications. *Eur Cytokine Netw* 2000;11(1):15-26.

Li J, Moran T, Swanson E, Julian C, Harris J *et al*. Regulation of IL-8 and IL-1beta expression in Crohn's disease associated *NOD2/CARD15* mutations. *Human Mol Genetics* 2004;13(16):1715-1725.

Liljemark WF, Bloomquist CG, Germaine GR. Effect of bacterial aggregation on the adherence of oral streptococci to hydroxyapatite. *Infect Immun* 1981;31(3):935-941.

Lindblom GB, Sjogren E, Hansson-Westerberg J, Kaijser B. *Campylobacter upsaliensis*, *C. sputorum sputorum* and *C. concisus* as common causes of diarrhoea in Swedish children. *Scand J Infect Dis* 1995;27:187-188.

Ling FC, Baldus SE, Khochfar J, Xi H, Neiss S *et al.* Association of COX-2 expression with corresponding active and chronic inflammatory reactions in Barrett's metaplasia and progression to cancer. *Histopathology* 2007;50:203-209.

Linton, D, Owen RJ, Stanley J. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res Microbiol* 1996;147:707-718.

Lochhead P, El-Omar EM. Gastric cancer. *Br Med Bull* 2008;85:87-100.

Locke GR 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastro-esophageal reflux: A population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997;112:1448-1456.

Lofdahl HE, Lu Y, Lagergren J. Sex-specific risk factor profile in oesophageal adenocarcinoma. *Br J Cancer* 2008;99(9):1506-1510.

Lu X, Yang L, Pei Z. Bacterium-macrophage interaction in gastroesophageal reflux disease. Abstract. *Lab Invest* 2006;86:113A.

Luo C, Xie S, Sun W, Li X, Cupples AM. Identification of a novel toluene-degrading bacterium from the candidate phylum TM7, as determined by DNA stable isotope probing. *App Env Microbiol* 2009;75(13):4644-4647.

Lund M, Nordentoft S, Pedersen K, Madsen M. Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. *J Clin Microbiol* 2004;42(11):5125-5132.

Macarthur M, Hold GL, El-Omar EM. Inflammation and cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. *Am J Physiol Gastrointest Liver Physiol* 2004;286:G515-G520.

MacCallum AJ, Harris D, Haddock G, Everest PH. *Campylobacter jejuni*-infected human epithelial cell lines vary in their ability to secrete interleukin-8 compared to *in vitro*-infected primary human intestinal tissue. *Microbiology* 2006;152:3661-3665.

Macfarlane GT, Blackett KL, Nakayama T, Steed H, Macfarlane S. The gut microbiota in inflammatory bowel disease. *Curr Pharm Des* 2009;15(13):1528-1536.

Macfarlane GT, Cummings JH. The colonic flora, fermentation and large bowel digestive function. In: Phillips SF, Pemberton JH, Shorter RR, ed. *The large intestine: physiology, pathophysiology, and disease*. Raven Press, New York, 1991:51-92.

Macfarlane GT, Macfarlane S, Gibson GR. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microb Ecol* 1998;35(2):180-187.

Macfarlane S, Furrie E, Macfarlane GT, Dillon JF. Microbial colonisation of the upper gastrointestinal tract in patients with Barrett's esophagus. *Clin Infect Dis* 2007;45(1):29-38.

Macfarlane S, Hopkins MJ, Macfarlane GT. Bacterial growth and metabolism on surfaces in the large intestine. *Microb Ecol Health Dis* 2000;Suppl 2:64-72.

Macfarlane S, Hopkins MJ, Macfarlane GT. Toxin synthesis and mucin breakdown are related to swarming phenomenon in *Clostridium septicum*. *Inf Immun* 2001;69(2):1120-1126.

Macfarlane S, Woodmansey EJ, Macfarlane GT. Colonisation of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Appl Environ Microbiol* 2005;71:7483-7492.

Malik-Kale P, Parker CT, Konkel ME. Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression. *J Bacteriol* 2008;190(7):2286-2297.

Manjunath S, Jankowski JAZ. Interaction of environmental factors in oesophageal carcinogenesis. *J R Coll Surg* 2000;45:259-276.

Mannell A, Plant M, Frolich J. The microflora of the oesophagus. *Ann R Coll Surg Engl* 1983;65:152-154.

Marcy Y, Ouverney C, Bik EM, Losekann T, Ivanova N *et al.* Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. *PNAS* 2007;104(29):11889-11894.

Marrero JM, de Caestecker JS, Maxwell JD. Effect of famotidine oesophageal sensitivity in gastro-oesophageal reflux disease. *Gut* 1994;35:447-450.

Marsh PD, Hunter JR, Bowden GH, Hamilton IR, McKee AS *et al*. The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. *J Gen Microbiol* 1983;129:755-770.

Marshall BJ, Warren JR. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;1(8336):1273-1275.

Martinez SD, Malagon IB, Garewal HS, Cui H, Fass R. Non-erosive reflux disease (NERD) – acid reflux and symptom patterns. *Aliment Pharmacol Ther* 2003;17:537-545.

Matsheka MI, Lastovica AJ, Elisha BG. Molecular identification of *Campylobacter concisus*. *J Clin Microbiol* 2001;39(10):3684-3689.

McColl KEL, Watabe H, Derakhshan MH. Role of gastric atrophy in mediating negative association between *Helicobacter pylori* infection and reflux oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma. *Gut* 2008;57:721-723.

McDermid AS, McKee AS, Marsh PD. A mixed-culture chemostat system to predict the effect of anti-microbial agents on the oral flora: preliminary studies using chlorhexidine. *J Dent Res* 1987;66:1315-1320.

McKee AS, McDermid AS, Ellwood DC, Marsh PD. The establishment of reproducible, complex communities of oral bacteria in the chemostat using defined inocula. *J Appl Bacteriol* 1985;59:263-275.

Mehra A, Lee KH, Hatzimanikatis V. Insights into the relation between mRNA and protein expression patterns: I. Theoretical considerations. *Biotechnol Bioeng* 2003;84(7):822-833.

Mercer CD, Wren SF, DaCosta LR, Beck IT. Lower esophageal sphincter pressure and gastroesophageal pressure gradients in excessively obese patients. *J Med* 1987;18(3-4):135-146.

Miller L, Berger T. Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett-Packard application note. *Hewlett-Packard*, PA, USA;1989:228-241.

Miller WG, Mandrell RE. Campylobacter in the food and water supply: Prevalence, outbreaks, isolation, and detection. In: Ketley J, Konkel ME, ed. *Campylobacter jejuni: New perspectives in molecular and cellular biology.* Norfolk, UK. Horizon Scientific Press, 2004;109–163.

Moayyedi P, Axon AT. Review article: gastro-oesophageal reflux disease-the extent of the problem. *Aliment Pharmacol Ther* 2005;22(suppl 1):11-19.

Moersch RN, Ellis FH, McDonald JR. Pathologic changes occurring in severe reflux esophagitis. *Surg Gynecol Obstet* 1959;108:476-484.

Moons LMG, Kusters JG, Bultman E, Kuipers EJ, van Dekken H *et al*. Barrett's oesophagus is characterized by a predominantly humoral inflammatory response. *J Pathol* 2005;207(3):269-276.

Morson BC, Belcher JR. Adenocarcinoma of the oesophagus and ectopic gastric mucosa. *Br J Cancer* 1952;6:127-130.

Mortensen NP, Kuijf ML, Wim Ang C, Schiellerup P, Krogfelt KA *et al.* Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect* 2009;11:988-994.

Mullis KB. The unusual origin of the polymerase chain reaction. *Sci Am* 1990;262(4):56-65.

Murray L, Sedo A, Scott M, McManus D, Sloan JM *et al.* TP53 and progression from Barrett's metaplasia to oesophageal adenocarcinoma in a UK population cohort. *Gut* 2006;55:1390-1397.

Musana AK, Resnick JM, Torbey CF, Mukesh BN, Greenlee RT. Barrett's esophagus: incidence and prevalence estimates in a rural Mid-Western population. *Am J Gastroenterol* 2008;103:516-524.

Nadkarni MA, Martin E, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002;148:257-266.

Nagri S, Hwang R, Anand S, Kurz J. Herpes simplex esophagitis presenting as acute necrotizing esophagitis ("black esophagus") in an immunocompromised patient. *Endsocopy* 2007;39(suppl 1):E169.

Nandurkar S, Talley NJ. Epidemiology and natural history of reflux disease. *Baillieres Best Pract Res Clin Gastroenterol* 2000;14(5):743-757.

Nardone G, Compare D. Epigenetic alterations due to diet and *Helicobacter pylori* infection in gastric carcinogenesis. *Expert Rev Gastroenterol Hepatol* 2008;2(2):243-248.

Nehra D, Howell P, Williams CP, Pye JK, Beynon J. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut* 1999;44:598-602.

Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ *et al*. Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA* 2006;295(13):1549-1555.

Oh DS, Hagen JA, Fein M, Bremner CG, Dunst CM *et al*. The impact of reflux composition on mucosal injury and esophageal function. *J Gastrointest Surg* 2006;10(6):787-797.

Olson WP, ed. *Automated microbial identification and quantitation*. Interpharm Press, IL, USA, 1996.

O'May GA, Reynolds N, Smith AR, Kennedy A, Macfarlane GT. Effect of pH and antibiotics on microbial overgrowth in the stomachs and duodena of patients undergoing percutaneous endoscopic gastrostomy feeding. *J Clin Microbiol* 2005a;43(7):3059-3065.

O'May GA, Reynolds N, Macfarlane GT. Effect of pH on an *in vitro* model of gastric microbiota in enteral nutrition patients. *Appl Environ Microbio*l 2005b;71(8):4777-4783.

On SLW. Confirmation of human *Campylobacter concisus* isolates misidentified as *Campylobacter mucosalis* and suggestions for improved differentiation between the two species. *J Clin Microbiol* 1994;32(9):2305-2306.

O'Riordan JM, Abdel-latif MM, Ravi N, McNamara D, Byrne PJ *et al.* Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Gastroenterol* 2005;100(6):1257-1264.

Osias G, Bromer M, Thomas RM. Esophageal bacteria and Barretts esophagus: a preliminary report. *Dig Dis Sci* 2004;49(2):228-236.

Overholt BF, Panjehpour M, Haydek JM. Photodynamic therapy for Barrett's esophagus: follow up in 100 patients. *Gastrointest Endosc* 1999;49(1):1-7.

Oyama K, Fujimura T, Ninomiya I, Miyashita T, Kinami S *et al*. A COX-2 inhibitor prevents the esophageal inflammation-metaplasia-adenocarcinoma sequence in rats. *Carcinogenesis* 2005;26(3):565-570.

Pajecki D, Zilberstein B, dos Santos MAA, Ubriaco JA, Quintanilha AG *et al*. Megaesophagus microbiota: a qualitative and quantitative analysis. *J Gastrointest Surg* 2002;6(5):723-729.

Palanca-Wessels MCA, Klingelhutz A, Reid BJ, Norwood TH, Opheim KE *et al.* Extended lifespan of Barrett's esophagus epithelium transduced with human telomerase catalytic subunit: a useful *in vitro* model. *Carcinogenesis* 2003;24(7):1183-1190.

Pandeya N, Williams G, Green AC, Webb PM, Whiteman DC. Alcohol consumption and the risks of adenocarcinoma and squamous cell carcinoma of the esophagus. *Gastroenterology* 2009;136:1215-1224.

Pandolfino JE, El-Serag HB, Zhang Q, Shah N, Ghosh SK *et al*. Obesity: a challenge to esophagogastric junction integrity. *Gastroenterology* 2006;130(3):639-649.

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C *et al*. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 2000;403(6770):665-668.

Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006;118:3030-3044.

Paster BJ, Gibbons RJ. Chemotactic response to formate by *Campylobacter concisus* and its potential role in gingival colonization. *Infect Immun* 1986;52(2):378-383.

Pathak SK, Basu S, Bhattacharyya A, Pathak S, Banerjee A *et al*. TLR4-dependent NF-kappa B activation and mitogen- and stress-activated protein kinase 1-triggered phosphorylation events are central to *Helicobacter pylori* peptidyl prolyl *cis*-, *trans*-isomerase (HP0175)-mediated induction of IL-6 release from macrophages. *J Immunol* 2006;177:7950-7958.

Patti MG, Goldberg HI, Arcerito M, Bortolasi L, Tong J *et al*. Hiatal hernia size affects lower esophageal sphincter function, esophageal acid exposure, and the degree of mucosal injury. *Am J Surg* 1996;171(1):182-186.

Paull A, Trier JS, Dalton MD, Camp RC, Loeb P *et al*. The histologic spectrum of Barrett's esophagus. *New Eng J Med* 1976;295(9):476-480.

Paunesku T, Mittal S, Protic M, Oryhon J, Korolev SV *et al*. Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. *Int J Radiat Biol* 2001;77(10):1007-1021.

Pei Z, Bini EJ, Yang L, Zhou M, Francois F *et al*. Bacterial biota in the human distal esophagus. *PNAS* 2004;101(12):4250-4255.

Pei Z, Yang L, Peek R, Levine SM, Pride DT *et al*. Bacterial biota in reflux oesophagitis and Barretts oesophagus. *World J Gastroenterol* 2005;11(46):7277-7283.

Peng DF, Razvi M, Chen H, Washington K, Roessner A *et al*. DNA hypermethylation regulates the expression of members of the Mu-class glutathione S-transferases and glutathione peroxidises in Barrett's adenocarcinoma. *Gut* 2009;58:5-15.

Periasamy S, Kolenbrander PE. Central role of early colonizer *Veillonella* sp. in establishing multispecies biofilm communities with initial, middle and late colonizers of enamel. *J Bacteriol* 2010;192(12):2965-2972.

Peters FT, Dleibeuker JH, Postma DS. Gastric asthma: a pathophysiological entity? *Scand J Gastroenterol* 1998;225(Suppl):19-23.

Pettersson GB, Bombeck Ct, Nyhus LM. The lower esophageal sphincter: mechanisms of opening and closure. *Surgery* 1980;88:307-314.

Piper MD, Daran-Lapujade P, Bro C, Regenberg B, Knudsen S *et al*. Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. *J Biol Chem* 2002;277:37001–37008.

Pisani P, Parkin DM, Munoz N, Ferlay J. Cancer and infection: estimates of the attributable fraction in 1990. *Cancer Epidemiol Biomarkers Prev* 1997;6:387-400.

Pitmann MS, Kelly DJ. Electron transport through nitrate and nitrite reductases in *Campylobacter jejuni*. *Biochem Soc Trans* 2005;33(1):190-192.

Pogacar MS, Mihaljevic RR, Klancnik A, Brumini G, Abram M *et al*. Survival of stress exposed *Campylobacter jejuni* in the murine macrophage J774 cell line. *Int J Food Microbiol* 2009;129(1):68-73.

Prach AT, MacDonald TA, Hopwood DA, Johnston DA. Increasing incidence of Barrett's esophagus: education, enthusiasm or epidemiology? *Lancet* 1997;350:933.

Reid BJ, Weinstein WM. Barrett's esophagus and adenocarcinoma. *Ann Rev Med* 1987;38:477-492.

Ribeiro U, Posner MC, Safatle-Ribeiro AV, Reynolds JC. Risk factors for squamous cell carcinoma of the oesophagus. *Br J Surg* 2005;83(9):1174-1185.

Richter JE, Bradley LA, Castell DO. Esophageal chest pain: current controversies in pathogenesis, diagnosis and therapy. *Ann Intern Med* 1989;110:66-78.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;11(2):94-100.

Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004;97:1166-1177.

Rowland IR. The role of the gastrointestinal microbiota in colorectal cancer. *Curr Pharm Des* 2009;15(13):1524-1527.

Russell J, Ward P. Adhesion and invasion of Hep-2 cells by *C. concisus* from children with diarrhoea. In: Lastovica AJ, Newell SDG, Lastovica EE, ed. *Campylobacter, Helicobacter and related organisms.* Cape Town: University of Capetown Press, 1998:327-330.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A *et al.* Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 2001;410(6827):471-475.

Sambrook J, Russell DW. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 2001;A2.2–A2.3.

Sandler RS, Everhart JE, Donowitz M, Adams E, Cronin K *et al.* The burden of selected digestive diseases in the United States. *Gastroenterology* 2002;122:1500-1511.

Sato F, Meltzer SJ. CpG island hypermethylation in progression of esophageal and gastric cancer. *Cancer* 2006;106(3):483-493.

Schulmann K, Sterian A, Berki A, Yin J, Sato F *et al.* Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's associated neoplastic progression and predicts progression risk. *Oncogene* 2005;25(25):4138-4148.

Schwerer B, Neisser A, Polt RJ, Bernheimer H, Moran AP. Antibody cross-reactivities between gangliosides and lipopolysaccharides of *Campylobacter jejuni* serotypes associated with Guillain-Barre syndrome. *J Endotox* 1995;2:395-403.

Scottish Intercollegiate Guidelines Network. Management of oesophageal and gastric cancer. SIGN NHS 2006:1-60.

Sebald M, Veron M. Teneur en bases de l'AND et classification des vibrions. *Ann Inst Pasteur* 1963;105:897-910.

Shaheen NJ, Crosby MA, Bozymski EM, Sandler RS. Is there publication bias in the reporting of cancer risk in Barrett's oesophagus? *Gastroenterology* 2000;119:333-338.

Shapiro JA. Thinking about bacterial populations as multicellular organisms. *Ann Rev Microbiol* 1998;52(1):81-104.

Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;31(1):27-36.

Sharma P, Sampliner RE, Camarago E. Normalization of esophageal pH with high-dose proton pump inhibitor therapy does not result in regression of Barrett's esophagus. *Am J Gastroenterol* 1997;92:582-585.

Shi G, Bruley des Varannes S, Scarpignato C, Le Rhun M, Galmiche JP. Reflux related symptoms in patients with normal oesophageal exposure to acid. *Gut* 1995;37:457-464.

Shiao YH, Rugge M, Correa P, Lehmann HP, Scheer WD. P53 alteration in gastric precancerous lesions. *Am J Pathol* 1994;144(3):511-517.

Signoretto C, Bianchi F, Burlacchini G, Sivieri F, Spratt D *et al*. Drinking habits are associated with changes in the dental plaque microbial community. *J Clin Microbiol* 2010;48(2):347-356.

Skirrow MB. Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *J Comp Pathol* 1994;111:113-149.

Smith JL, Bayles DO. The contribution of cytolethal distending toxin to bacterial pathogenesis. *Crit Rev Microbiol* 2006;32(4):227-248.

Socansky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;25(2):134-144.

Solaymani-Dodaran M, Coupland C, Logan RFA. Risk of oesophageal cancer in Barrett's oesophagus and in gastro-oesophageal reflux. *Gut* 2003;52:A20.

Spechler SJ. Barrett’s esophagus. *Gastroenterologist* 1994;2(4):273-284.

Spechler SJ. Clinical practice. Barrett's esophagus. *N Engl J Med* 2002;346(11):836-842.

Stager CE, Davis JR. Automated systems for identification of microorganisms. *Clin Microbiol Rev* 1992;5(3):302-327.

Stein HJ, Barlow AP, De Meester TR, Hinder RA. Complications of gastroesophageal reflux disease – role of the lower esophageal sphincter, esophageal acid and acid/alkaline exposure, and duodenogastric reflux. *Ann Surg* 1992;216(1):35-43.

Stewart JA, Chadwick VS, Murray A. Investigations into the influence of host genetics on the predominant eubacteria in the faecal microflora of children. *J Med Microbiol* 2005;54:1239-1242.

Stoner GD, Kaighn ME, Reddel RR, Resau JH, Bowman D *et al.* Establishment and characterization of SV40 T-antigen immortalized human esophageal epithelial cells. *Cancer Res* 1991;51:365-371.

Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 2002;56:187-209.

Strachan NJC, Watson RO, Novik V, Hofreuter D, Ogden ID *et al*. Sexual dimorphism in campylobacteriosis. *Epidemiol Infect* 2008;136:1492-1495.

Suzuki H, Iijima K, Scobie G, Fyfe V, McColl KEL. Nitrate and nitrosative chemistry within Barrett's oesophagus during acid reflux. *Gut* 2005;54:1527-1535.

Swisher SG, Deford L, Merriman KW, Walsh GL, Smyth R, *et al*. Effect of operative volume on morbidity, mortality, and hospital use after esophagectomy for cancer. *J Thorac Cardiovasc Surg*. 2000;119:1126-1132.

Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA* 2002;99:3740-3745.

Tang G, Yip HK, Cutress TW, Samaranayake LP. Artificial mouth model systems and their contribution to caries research: a review. *J Dent* 2003;31(3):161-171.

Tanner AC, Haffer C, Bratthall GT, Visconti RA, Socransky SS. A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol* 1979;6:278-307.

ten Cate JM. Biofilms, a new approach to the microbiology of dental plaque. *Odontology* 2006;94(1):1-9.

ter Linde JJM, Llang H, Davis RW, Steensma HY, van Dijken JP *et al*. Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *J Bacteriol* 1999;18(24):7409-7413.

Thong-Ngam D, Tangkijvanich P, Lerknimitr R, Mahachai V, Theamboonlers A *et al*. Diagnostic role of serum interleukin-18 in gastric cancer patients. *World J Gastroenterol* 2006;12(28):4473-4477.

Tileston W. Peptic ulcer of the esophagus. *Am J Med Sci* 1906;132:240-265.

Todd JA, Johnston DA, Dillon JF. The changing spectrum of gastroesophageal reflux disease. *Eur J Can Prev* 2002;11:215-219.

Townes JM, Deodhar AA, Laine ES, Smith K, Krug HE *et al.* Reactive arthritis following culture-confirmed infections with bacterial enteric pathogens in Minnesota and Oregon: a population-based study. *Ann Rheum Dis* 2008;67(12):1689-1696.

Tselepis C, Perry I, Dawson C, Hardy R, Darnton SJ *et al*. Tumour necrosis factor-α in Barrett's oesophagus: a potential novel mechanism of action. *Oncogene* 2002;21(39):6071-6081.

Tu QV, McGuckin MA, Mendz GL. *Campylobacter jejuni* response to human mucin MUC2: modulation of colonization and pathogenicity determinants. *J Med Microbiol* 2008;57:795-802.

Uzoigwe C. *Campylobacter* infections of the pericardium and myocardium. *Clin Microbiol Infect* 2005;11:253-255.

Vandamme P, Falsen E, Rossau R, Hoste B, Segers P *et al*. Revision of *Campylobacter, Helicobacter,* and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* 1990;41:88-103.

van Laethem JL, Jagodzinski R, Peny MO, Cremer M, Deviere J. Argon plasma coagulation in the treatment of Barrett's high-grade dysplasia and *in situ* adenocarcinoma. *Endoscopy* 2001;33(3):257-261.

van Putten JPM, van Alphen LB, Wosten MMSM, de Zoete MR. Molecular mechanisms of *Campylobacter* infection. *Curr Top Microbiol Immunol* 2009;337:197-229.

van Soest EM, Dieleman JP, Siersema, Sturkenboom MC, Kuipers EJ. Increasing incidence of Barrett's oesophagus in the general population. *Gut* 2005;54:1062-1066.

Veuglers PJ, Porter GA, Guernsey DL, Casson AG. Obesity and lifestyle factors for GORD, Barrett's and esophageal adenocarcinoma. *Dis Esophagus* 2006;19:321-328.

Walter J, Hertel C, Tannock GW, Lis CM, Munro K *et al.* Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 2001;67:2578-2585.

Wang B, Kraig E, Kolodrubetz D. Use of defined mutants to assess the role of the *Campylobacter rectus* S-Layer in bacterium-epithelium cell interactions. *Infect Immun* 2000;68(3):1465-1473.

Watson A. Barrett's oesophagus – 50 years on. *Br J Surg* 2000;87:529-531.

Watson A, Heading RC, Shepherd NA. BSG guidelines for the diagnosis and management of Barrett's columnar-lined oesophagus (CLO). *Br Soc Gastroenterol* 2005.

Welch DF. Applications of cellular fatty acid analysis. *Clin Microbiol Rev* 1991;4(4):422-438.

Westhoff B, Brotze S, Weston A, McElhinney C, Cherian R *et al*. The frequency of Barrett's esophagus in high-risk patients with chronic GERD. *Gastrointest Endosc* 2005;61(2):226-231.

Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* 2007;20(4):593-621.

Wick LM, Quadroni M, Egli T. Short- and long-term changes in proteome composition and kinetic properties in a culture of *Escherichia coli* during transition from glucose-excess to glucose-limited growth conditions in continuous culture and vice versa. *Environ Microbiol* 2001;3:588–599.

Wilson KT, Crabtree JE. Immunology of *Helicobacter pylori*: insights into the failure of the immune response and perspectives on vaccine studies. *Gastroenterology* 2007;133:288-308.

Wink DA, Fellisch M, Vodovotz Y, Fukuto J, Grisham MB. The chemical biology of nitric oxide. In: Gilbert DL, Goton CA, ed. *Reactive oxygen species in biological systems.* New York: Kluwer Academic Plenum Publishers, 1999;245-292.

Wisen O, Rossner S, Johansson C. Impaired pancreatico-biliary response to vagal stimulation and to cholecystokinin in human obesity. *Metabolism* 1988;37:436-441.

Wolfsen HC, Woodward TA, Raimondo M. Photodynamic therapy for dysplastic Barrett's esophagus and early esophageal adenocarcinoma. *Mayo Clin Proc* 2002;77(11):1176-1181.

World Health Organisation (WHO, 2010), [cited July 2010]; Available from: http://www.who.int/cancer/en/

Yang L, Lu X, Nossa CW, Francois F, Peek RM *et al*. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. *Gastroenterology* 2009;137:588-597.

Zaninotto G, De Meester TR, Schwizer W, Johansson KE, Cheng SC. The lower esophageal sphincter in health and disease. *Am J Surg* 1988;155:104-111.

Zheng J, Meng J, Zhao S, Singh R, Song W. *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin and Toll-like receptor mediated activation of NF-kB. *Infect Immun* 2008;76(10):4498-4508.

Zoetendal EG, Akkermans AD, Akkermans-van Vliet WM, de Visser JAGM, de Vos WM. The host genotype affects the bacterial community in the human gastrointestinal tract. *Microb Eco Health Dis* 2001;13:129-134.

Zoetendal EG, Vaughan EE, de Vos WM. A microbial world within us. *Mol Microbiol* 2006;59(6):1639-1650.